ENHANCING CHICKEN MUCOSAL IGA RESPONSE AGAINST *CLOSTRIDIUM PERFRINGENS* α-TOXIN

A Dissertation

by

CHANG-HSIN CHEN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2012

Major Subject: Poultry Science

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ABSTRACT

Enhancing Chicken Mucosal IgA Response against *Clostridium perfringens* α-toxin.

(August 2012)

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Necrotic enteritis (NE) is an economically important enteric disease of broiler chickens primarily caused by α-toxin (Cpα) secreted by *C. perfringens* type A. Mice immunized with recombinant C-terminal domain of Cpα ($Cp\alpha_{CD}$) had transient and fewer localized lesions upon challenge with *C. perfringens* type A. These results demonstrate the usefulness of $Cp\alpha_{CD}$ as an immunogen for vaccine development against NE for chickens. Chicken CD40 (chCD40) is mainly expressed on the surface of chicken antigen-presenting cells (APCs), and the interaction of chCD40 and chCD40L (natural ligand for chCD40) provides crucial activation signals for chicken B-cells. A hypothesis was proposed that *in ovo* vaccination with an adenovirus-vectored $Cp\alpha_{CD}$ vaccine capable of targeting immunogen to APCs through the CD40 pathway will improve protection against NE in chickens. One agonistic monoclonal anti-chCD40 antibody (designated 2C5) was produced and characterized. 2C5 not only detected expression of chCD40 on chicken APCs, but also induced NO synthesis in chicken HD11 macrophages and enhanced proliferation of serum-starved chicken DT40 B-cells. This demonstrated substantial functional equivalence of 2C5 with chCD40L. The potential of 2C5 as an immunological adjuvant was further assessed by targeting a hapten to chicken APCs in hopes of enhancing an effective IgG response. Seven-week old chickens were immunized

subcutaneously once with a complex consisting of 2C5 and peptide, and relative quantification of the peptide-specific IgG response showed that this complex was able to elicit a strong IgG response as early as four days post-immunization. This demonstrates that CD40-targeting antigen to chicken APCs can significantly enhance antibody responses and induce immunoglobulin isotype-switching. An agonistic anti-chCD40 single-chain variable fragment (designated DAG1) was combined with an adenoviral delivery system to create a vaccine, Ad-(DAG1-Cpα_{CD}-FLAG), for *in ovo* administration. The efficacy of *in ovo* vaccination of broilers with Ad-(DAG1-Cpα_{CD}-FLAG) in controlling NE was evaluated by *C. perfringens* type A challenge at 18 days post-hatch. Neither statistically significant IgA / IgG response nor protection against *C. perfringens* type A challenge was found in the vaccinated birds. These preliminary data suggest that a super-optimal dose of Ad-(DAG1-Cpα_{CD}-FLAG) may be the main issue, because Cpα-specific B-cells may undergo apoptosis through the CD40 pathway.

DEDICATION

To my deceased grandmother,

To my parents and family members,

To my friends,

For their endless love, support, patience, and encouragement.

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The experience of my doctoral study was very much a time of intensive learning, not only in the field of poultry immunology but also in many other aspects of life. I hope that I have become wiser and more mature as a result of this experience. My research has taken a great amount of input of effort, perseverance, and skill, and many people have contributed to this dissertation. First of all, I would like to thank my advisor, Dr. Berghman, and all my committee members, Dr. Farnell, Dr. Moore, Dr. Ruiz-Feria, and Dr. Waghela. I also want to thank for Dr. Hargis and Dr. Mwangi for their help and support during the completion of my last experiment, the most challenging one in my dissertation.

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CHAPTER I

INTRODUCTION

Necrotic enteritis (NE) is an economically important enteric disease of modern chickens primarily caused by infection with *Clostridium perfringens* type A (Cooper and Songer, 2009). The acute form of NE in a broiler flock can account for 1% losses per day, for several consecutive days during the last weeks of the rearing period (Cooper and Songer, 2009). Combined with the broiler meat production estimate recently, the cost of NE including clinical and subclinical infections was close to $2 billion US dollar worldwide (Lee et al., 2011). In addition, some strains of *C. perfringens* also produce enterotoxins that cause food-borne disease in humans (Collie and McClane, 1998).

NE is suspected to occur under certain predisposing conditions when *C. perfringens* type A, and to a lesser extent type C multiply in excess numbers and secrete toxins in small intestine that was previously damaged by *Eimeria* (Songer, 1996). Due to the voluntary or legally required withdrawal of the use of antibiotic growth promoters in feed, few tools and strategies are available for efficient prevention and control of NE in chickens. The economic toll of this disease is only expected to rise, as therapeutic agents become increasingly unavailable, and management practices are altered (Cooper and Songer, 2009). Vaccine is considered as the most promising method for prevention of NE, but no efficient vaccine against NE for chickens is available on the market to date (Lee et al., 2011).

C. perfringens type A produces several exotoxins, and the predominant one is α-toxin, which belongs to phospholipase superfamily (Titball et al., 1991). *C. perfringens*α-toxin

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This dissertation follows the style and format of *Journal of Immunological Methods.*

(Cpα) is one of the most potent toxic phospholipases characterized to date (Titball et al., 1993). *C. perfringens* α-toxin (Cpα) is one of the most potent toxic phospholipases characterized to date (Titball et al., 1993). Cpα has been implicated as the major cause of lesions associated with NE (Al-Sheikhly and Truscott, 1977a), and is also known as contributor in the pathogenesis of a variety of diseases, such as gas gangrene in humans, in different animal species (Cooper and Songer, 2009). Several studies have shown that immunization with crude Cpα as immunogen can induce protection against diseases caused by *C. perfringens*. Mice immunized intraperitoneally with a recombinant C-terminal domain of Cpα (amino acid 247-370) had transient and localized lesions compared to sham immunized mice upon challenge with *C. perfringens* type A. At a high dose challenge (3.74 x 10^9 cfu), there were no survivors in the control group, whereas 90% of mice in the immunized group survived. This demonstrates the usefulness of the C-terminal domain of the Cpα as an immunogen for development of vaccine against NE for chickens (Stevens et al., 2004).

The interaction between CD40 [mainly expressed by antigen presenting cells (APCs)] and CD154 (expressed by T-cells) mediates the major signal in T-cell help to B-cells (Banchereau et al., 1994). It drives or co-stimulates activation, proliferation, differentiation, and antibody production on B-cell (Xu and Song, 2004). Using CD40-targeted antigen delivery, up to 1000-fold increased antibody responses were reported in mice (Barr et al., 2003). Agonistic anti-CD40 antibodies not only target antigen delivery and activate B-cells, but also induce antibody class-switching (Barr et al., 2006). Murine and human naïve B-cells can be activated with agonistic anti-CD40 antibody or CD40L to undergo class switch recombination on immunoglobulin gene *in vitro* (Kracker and Radbruch, 2004).

Immunoglobulin class switching through CD40 pathway is most crucial for mucosal immunity because IgA is readily transported across the intestinal mucosa and is endowed with effector properties that are critical for the local humoral immune response (Zan et al., 1998). Recently, CD40-mediated enhancement of isotype-switched antibody responses against a hapten in chickens was evaluated (Chen et al., 2012). Adjuvant effects of agonistic anti-chicken CD40 antibody were observed while a hapten was biologically complexed to it. Significant hapten-specific and isotype-switched antibody responses were observed only four days after a single immunization with very low amount of antigen, and significant hapten-specific antibody response had been maintained for two weeks.

Using recombinant replication-defective human adenoviruses, such as adenovirus type 5 (Ad5) has many advantages for vaccine development because vectored adenovirus vaccine can be generated rapidly and mass-produced at low cost (Avakian et al., 2007). First, they are able to introduce foreign gene into a wide spectrum of host cells. Second, transduced gene is transiently maintained and expressed for several weeks in host cells, which produces reasonable immunity against the foreign protein. Finally, despite its human origin, Ad5 is able to target foreign genes in a wide range of animal species, even those in which Ad5 cannot replicate (Ali et al., 1994). Transduction of chicken embryotic cells *in vivo* by replication-defective Ad5 was reported for the first time in 1995 (Adam et al., 1995). *In ovo* delivery of vectored adenovirus vaccines makes it possible to administer a wide variety of pathogen derived antigens in large scale and deliver high potency in a single-dose regimen, which does not interfere with epidemiological surveys of natural infections. At the experimental level, antigen delivery by Ad5 can also avoid the interference by maternal immunity while *in ovo* administration route is required (Breedlove et al., 2011).

In ovo vaccination plays an important role in protecting chickens from several diseases (Josefsberg and Buckland, 2012). *In ovo* injection system vaccinates up to 70,000 embryonated chicken eggs per hour in a more uniform and precise manner than post-hatch mass vaccination methods (Perez and Ronchi, 1996). Today, approximately 90% of broiler chickens grown in the USA are vaccinated *in ovo* 2-3 days prior to hatch at the time when eggs are transferred from the incubator to the hatcher (Avakian et al., 2007). Antigen delivery by adenovirus to the amniotic fluid of the embryo is highly effective because during E17-19, the embryo imbibes the amniotic fluid, and vectored adenovirus can rapidly transduce cells on the respiratory tract (Sharma et al., 2002) and the digestive system (Kapczynski et al., 2003). These results in highly efficient antigen delivery to the mucosa-associated lymphoid tissue (MALT) as well as Peyer's patches, the most important secondary lymphoid organ for mammals, proposed for chickens, in mucosal immunity. Exposure to antigen drives the maturation of avian MALT (Reese et al., 2006) and suggests that *in ovo* immunization may accelerate the maturation of embryonic Bronchus-Associated Lymphoid Tissue (BALT) (Moyron-Quiroz et al., 2004). In addition, the hexon protein on adenovirus is highly immunogenic and confers adjuvant activity to exogenous antigens (Molinier-Frenkel et al., 2002).

The goal of this study is to produce and evaluate an *in ovo* delivered vectored adenovirus vaccine for the prevention and control of *C. perfringens*-associated morbidity and mortality in poultry. By fusing in-frame, the single-chain variable fragment (scFv) to another molecule, a moiety can be achieved that is specific for a particular target with an enhanced function (Todorovska et al., 2001). One agonistic anti-CD40 scFv (DAG1) was fused to $Cp\alpha_{CD}$ for a targeted, more efficient delivery to APCs in order to enhance $Cp\alpha_{CD}$ specific

antibody response. This DAG1-Cpα_{CD} chimera was further cloned into an adenoviral vector for adenovirus production for *in ovo* vaccination. This *in ovo* vaccination with a non-replicating human adenovirus vector expressing DAG1-Cpα_{CD} is a novel concept for induction of *C. perfringens* α-toxin specific IgA responses because it will direct active uptake of the Cpα_{CD} by APCs, and simultaneously provide well-characterized signals for both APC activation and IgA switch factor through CD40 signaling. This CD40-targeting strategy will improve clinical efficacy of a *C. perfringens* α-toxin vaccine and this outcome will be significant because an efficacious vaccination strategy against NE will contribute to an increase in the efficiency of poultry production systems. More broadly, this vaccine delivery system will provide an opportunity for a systematic evaluation of further modifications that will enhance the immunogenicity of the Cpα vaccine.

CHAPTER II

REVIEW OF LITERATURE

Necrotic enteritis (NE)

The poultry industry has grown conspicuously and transformed itself into a highly specialized field over the past 40 years; substantial economic investment, like vaccination, is required (Chapman et al., 2003; Cooper and Songer, 2009). Higher efficiency of growth, feed conversion, and meat yield of poultry is highly improved by genetic selection (Cooper and Songer, 2009). These advances result in higher producing rates of poultry and save 65-70% of total cost investing in feed (Cooper and Songer, 2009). However, the profit of poultry industry is greatly affected by unexpected high mortality in flocks caused by infectious diseases. One of these is necrotic enteritis (NE), an economically important enteric disease with high mortality rate in poultry primarily caused by *Clostridium perfringens* type A (Craven et al., 1999), which is estimated to cost the international poultry industry more than $2 billion US dollar per year (McReynolds et al., 2004). The occurrence of NE in poultry has been well documented since it was first diagnosed in 1961. During the 1970s, Al-Sheikhly and colleagues suggested that α-toxin produced by *C. perfringens* was the main virulence factor involved in pathogenesis of NE (Al-Sheikhly and Truscott, 1977a; Al-Sheikhly and Truscott, 1977c; Al-Sheikhly and Truscott, 1977b). This suggestion was based on the observations that α-toxin is a major secreted toxin from *C. perfringens,* and filtered culture media from *C. perfringens* can still cause necrotic lesions typical of NE in the gastrointestinal tract of chickens (Al-Sheikhly and Truscott, 1977c; Al-Sheikhly and Truscott,

1977b). Subsequent reports also demonstrate that α-toxin was the prerequisite virulence factor for development of NE (Al-Sheikhly and Truscott, 1977a; Fukata et al., 1988).

Because of the suddenly increased mortality found in affected chickens, clinical or subclinical NE has devastating effects on poultry industry (Gholamiandehkordi et al., 2007). Clinical signs in chickens suffering from clinical NE include depression, decreased feed intake, reluctance to move, ruffled feathers, and diarrhea (Hermans and Morgan, 2007), and affected ones can die acutely. Daily mortality rates can attain 1% per flock. Without medical treatment, 10-40% of chickens in an affected flock may die. Subclinical NE (milder form) is more common in the poultry industry, and may exert a significantly negative impact on production. Subclinical NE often goes undetected, and hence affects the animal welfare when medical treatment is not provided. Due to damage of the intestinal mucosa, and subsequent decreased digestion and absorption, sick chickens exhibit decreased weight gain, and increased feed conversion ratio. The productivity of each flock can be greatly affected (Kaldhusdal et al., 1999). The mildest form of NE induces no visible illness on chickens but is associated with temporarily reduced weight gain, impaired feed conversion ratio, and increased condemnation rates at slaughter due to liver lesions.

C. perfringens

C. perfringens is a gram-positive, spore-forming, anaerobic enteropathogen that is naturally found in soil, water, sewage, and intestinal environments of humans and animals (Hatheway, 1990). Genomic analysis revealed that *C. perfringens* lacks the genetic machinery to produce 13 essential amino acids, and can only obtain them *in vivo* by lysing host cells via actions of exotoxins, which are phospholipase or pore-forming enzymes (Myers

et al., 2006). Reports indicate that more than 17 toxins or potentially toxic exoproteins are produced by *C. perfringens,* which are classified into toxigenic serotypes A, B, C, D, and E based on their ability to produce five major toxins: α, β, ι, ε, and θ (Brynestad and Granum, 2002). *C. perfringens* is the causative agent of a number of diseases, including NE, gas gangrene, and type A diarrhea in both humans and chickens (Brynestad and Granum, 2002). In fact, *C. perfringens* is one of the most frequently isolated bacterial pathogens in food borne disease outbreaks, after pathogens such as Campylobacter and Salmonella (Buzby and Roberts, 1997). Frequent fatal outbreaks have been attributed to *C. perfringens* food poisoning (Briggs et al., 2011). The spore-forming feature of *C. perfringens* and its ability to multiply and survive in food at a range of temperatures, have led to detection of this organism in a multitude of raw and processed foods, namely meat, meat products, and spices (Brynestad and Granum, 2002).

C. perfringens can also survive under variable environmental conditions in extended periods in poultry farms (Van Immerseel et al., 2004). Colonization of the avian intestinal tract by *C. perfringens* appears to be a very early event in the life of the animals, and can be transmitted within an integrated operation (Craven et al., 2003). Although several subtypes of *C. perfringens* may be presented in healthy chickens and even in those with subclinical symptoms, subtypes isolated from sick chickens in different flocks may differ. *C. perfringens* type A is reported to be the main pathogen proliferating in chickens with clinical NE (Yoo et al., 1997) and has also been isolated from livers with cholangiohepatitis in sick chickens (Kaldhusdal et al., 2001; Lovland and Kaldhusdal, 2001; Lovland et al., 2004). *C. perfringens* type C is also reported to be associated with NE, but most cases appear to be caused by type A (Thompson et al., 2006).

The two most well-known forms of *C. perfringens* associated disease in chickens are NE and cholangiohepatitis (Lovland and Kaldhusdal, 1999). Cholangiohepatitis is usually detected on the carcass at slaughter houses or as an incidental finding during exploratory surgery on chickens or necropsy of chickens collected during the rearing period. Clinical and subclinical NE are often found in chickens affected by cholangiohepatitis (Cooper and Songer, 2009). NE is most commonly found in broilers, young replacement broiler breeders, and young meat turkeys. Broilers at two to five weeks of age are most frequently affected, and *C. perfringens* is also found frequently in intestinal contents of broiler from 2 weeks of age and throughout the rearing period though (Long and Truscott, 1976). NE is also regularly found in layers, mostly in pullets and young layers kept on litter (Lovland and Kaldhusdal, 1999). Low level maternal immunity against *C. perfringens* is associated with an increased risk of NE in broilers. Broiler chicks are particularly susceptible to NE when maternal antibodies have waned and the level of actively produced specific antibodies is still low, especially those broiler chicks from young parent hens have lower levels of maternal antibodies against *C. perfringens* (Lovland et al., 2004).

C. perfringens α-toxin (Cpα)

α-toxin is encoded by the phospholipase gene reported to be highly conserved in *C. perfringens* strains isolated from sick chickens, with variations in only nine out of 397 deduced amino acid positions. α-toxin is a zinc metallophospholipase possessing activities of both phospholipase C and sphingomyelinase (Saint-Joanis et al., 1989). It contains an α-helical N-terminal domain ($Cp\alpha_{ND}$, residues 1–246) encompassing the active site (Titball et al., 1993) and a β-sandwich C-terminal domain ($Cp\alpha_{CD,}$ residues 256–370) considered

essential for membrane binding (Titball et al., 1991; Titball et al., 1993; Nagahama et al., 1998; Naylor et al., 1998). α-toxin destroys cell membranes by oxidation and hydrolysis of membrane phospholipids, and also enters the blood stream, causing systemic effects and death (Thompson et al., 2006). $Cp\alpha_{ND}$ exhibits a strong sequence homology to the entire *Bacillus cereus* phospholipase C (PLC), which is non-toxic. The recombinant $Cp\alpha_{ND}$ retains its PLC activity but loses its hemolytic or lethal activity with a markedly reduced sphingomyelinase activity. However, these activities are restored in the presence of recombinant $Cp\alpha_{CD}$. This complementation occurs probably due to hydrophobic interactions of the two domains (Titball et al., 1993). The $Cp\alpha_{CD}$ by itself has no enzymatic/toxic activities but is involved in cell-membrane disruption (Titball et al., 1993). Neither $Cp\alpha_{ND}$ nor $Cp\alpha_{CD}$ is cytotoxic by itself, whereas α-toxin is cytotoxic to mouse lymphocytes. Antiserum raised against $Cp\alpha_{CD}$ has equal potency to antiserum against α-toxin in neutralizing the PLC and hemolytic activities of the holoenzyme *in vitro*, leading to the conclusion that the $Cp\alpha_{CD}$ is a good immunogen for vaccine development (Stevens et al., 2004).

While α-toxin was previously believed to be the main virulence factor involved in NE pathogenesis in chickens (Al-Sheikhly and Truscott, 1977c; Fukata et al., 1988; Williamson and Titball, 1993; Thompson et al., 2006), the recent identification of necrotic enteritis toxin B (NetB) and hypothetical protein (HP) produced by *C. perfringens,* which is related to NE, has called the validity of this concept into question (Keyburn et al., 2006; Keyburn et al., 2008; Kulkarni et al., 2008; Olkowski et al., 2008). Although NetB / HP was demonstrated to be critical for the ability of *C. perfringens* to cause NE and its identification provides a significant opportunity for the development of novel vaccines against NE in poultry, most of

the solid evidence of the role of NetB / HP pathogenesis in NE is currently still under investigation (Sumners et al., 2012).

Pathogenesis of NE

Although the pathogenesis of NE is not completely understood, the presence of *C. perfringens* in the intestine alone is insufficient to induce NE, and two requirements for induction of NE in chickens have been proposed (Al-Sheikhly and Truscott, 1977a). First, preexisting damage to the intestinal mucosa caused by predisposing factors, such as coccidiosis. Second, higher numbers of *C. perfringens* than would typically exists in normal flora of the chicken intestine. If these two requirements are fulfilled sequentially, development of lesions often starts at the tips of the villi. The damaged villi are observed where *C. perfringens* adhere, proliferate, denude lamina propria, and induce coagulative necrosis. Attraction and lysis of heterophils can cause further tissue necrosis, as makes bacterial proliferation proceed rapidly.

Mucosal damage induced by *Eimeria* parasites is reported to be the most important predisposing factor because this damage on epithelial surface of the intestinal tract allows for the establishment of *C. perfringens* (Johansson and Sarles, 1948; Nairn and Bamford, 1967; Helmboldt and Bryant, 1971). Although coccidia do not induce lesions in the small intestine where lesions of NE usually develop, caecal coccidiosis may increase the shedding of *C. perfringens* resulting in the contamination of the rearing environment (Baba et al., 1992).

The pathogenic mechanism of cholangiohepatitis, the most common *C. perfringens* associated liver disease, has been reproduced experimentally by inoculation with *C. perfringens* / *C. perfringens* toxins into the hepatoenteric bile duct (Nairn and Bamford, 1967;

Kaldhusdal et al., 2001). These results suggest that *C. perfringens* / *C. perfringens* toxins may reach the liver and lead to bile stasis and inflammation of the biliary tract. The incidence of cholangiohepatitis shows a striking parallel with the incidence of NE, which suggests that this is another manifestation of clinical / subclinical NE, and that *C. perfringens* is the etiology of the disease / lesions (Lovland and Kaldhusdal, 2001; Van Immerseel et al., 2004).

Horizontal transmission has been considered as the significant means of dissemination of *C. perfringens*, but recent findings suggest that even vertical transmission is possible (Shane et al., 1984). *C. perfringens* may be transmitted between facilities within an integrated broiler chicken operation. Molecular subtyping of *C. perfringens* isolates suggests that organisms contaminating the processed product could originate from the poultry environment prior to grow out (Dhillon et al., 2004).

Controlling NE

The predisposing factors leading to outbreaks of NE in chickens are exceedingly important (Van Immerseel et al., 2004). Overcrowding of chickens kept on litter, inadequate hygiene routines, wet litter problems, and feed composition are the main issues (Nairn and Bamford, 1967; Helmboldt and Bryant, 1971). The risk of NE is low when chickens are kept on wire floor that minimizes contact with feces, but NE outbreaks occasionally appear even in chicken kept on wire floors (Craven et al., 2001; Craven et al., 2003; Van Immerseel et al., 2004). Another predisposing factor of NE outbreak is the feed, which may be contaminated with *C. perfringens* despite the pelleting process or heat treatment (Nairn and Bamford, 1967; Eleazer and Harrell, 1976; Hofacre et al., 1986). Sudden changes in rations of feed component can alter the intestinal pH and native microbial population, and give rise to

opportunistic bacteria such as *C. perfringens* (Apajalahti et al., 2001). Several studies indicate that maize/wheat based feed may contribute to the prevention of NE because these feeding ingredients have been reported to lower the pH of gizzard contents and reduce intestinal counts of *C. perfringens* (Prescott, 1979; Cooper et al., 2009). Some diets containing indigestible, water soluble, non-starch polysaccharides known to increase the viscosity of the ingesta, such as barley, wheat, maze, and rye, have also been incriminated as predisposing factors for NE (Branton et al., 1997). The source of dietary fat can also be important. Intestinal counts of *C. perfringens* in broilers fed with food supplied with soy-oil were lower than those with lard/tallow (Williams, 2005). High levels of crude dietary proteins may also be a risk factor (Truscott and Al-Sheikhly, 1977; Dahiya et al., 2005) because these differences between protein ingredients have been correlated with dietary glycine levels. Dietary glycine level and *C. perfringens* counts experiments suggest high levels of glycine are a predisposing factor for NE (Dahiya et al., 2005). Dietary animal protein was reported to be associated with a higher risk of NE than other relevant protein sources from plants (Long, 1973; Van Immerseel et al., 2004; Gholamiandekhordi et al., 2006; Olkowski et al., 2006), and especially fish meal is considered a frequent source of contamination of *C. perfringens* (Drew et al., 2004).

Controlling NE was commonly achieved in a preventive manner, thus antimicrobial drugs are added in feed or water. However, the use of antibiotics in this manner was banned by the European Union because of the concern about that routine prophylactic use of antibiotics may contribute to problems of antimicrobial resistance (Emborg et al., 2003; Glassmeyer et al., 2005). This policy makes NE become much more widespread (from 4% to 12.4%), especially in Western Europe, and investigation of alternative control measures are

highly needed (Jiang et al., 2009). Increasing restrictions on the use of in-feed antimicrobials in Europe and other regions promotes the interest in non-antimicrobial measures against NE. Developing a non-antimicrobial preventive strategy against this disease is one of today's major challenges with regard to poultry health and welfare.

Non-antimicrobial feed additives and oral treatments have been proposed as potential preventive measures for NE, these include probiotics or competitive exclusion products, plant-derived products, non-digestible compounds assumed to promote a healthy gut flora, supplementary enzymes and acids (Casewell et al., 2003; Collier et al., 2003; Drew et al., 2004; Van Immerseel et al., 2004). Promising effects in terms of suppressing growth of *C. perfringens*, and in some cases prevention of NE, have been reported for products within all of these groups of oral treatments but so far no single alternative measure as effective as the in-feed antimicrobials has been found. Based on naturally occurring antibodies or maternal vaccination against *C. perfringens*, some studies suggest that specific humoral immune response against α-toxin provides a good degree of protection against NE (Zekarias et al., 2008; Cooper et al., 2009; Jiang et al., 2009). Vaccines against NE in poultry are not yet available but experimental work suggests that vaccination may become a significant contribution to the preventive efforts. Several studies indicate that the crude α-toxin as an immunogen induces good protection (Hoang et al., 2008; Zekarias et al., 2008).

CD40

CD40, an integral membrane glycoprotein of the TNF-receptor super family, is expressed mainly on professional antigen-presenting cells (APCs), including B-cells, macrophages, and dendritic cells (van Kooten and Banchereau, 2000). However, in mammals,

expression of CD40 is not restricted to APCs. Reports indicate that high levels of CD40 can also be detected on a wide range of human cancer cells from the bladder, breast, and ovary (Sabel et al., 2000; Hill et al., 2005).

The natural ligand for CD40 is CD154 (CD40L) and it is transiently expressed on activated $CD4^+$ T-cells (Armitage et al., 1992). In addition, platelets are also reported to be a major source of secreted CD40L. The engagement of the T-cell bound CD154 ligand to the CD40 receptor on APCs provides the critical signal required for optimal APC activation which results in up-regulation of MHC and CD40 molecules, expression of CD80/86, and secretion of cytokines (Grewal and Flavell, 1996; Noelle, 1996). These outcomes are critical for optimal priming and expansion of antigen-specific effector and memory T-cells, B-cell response, and immunoglobulin class switching (Gordon and Pound, 2000). The importance of CD40–CD40L ligation has been clearly demonstrated by the human hyper-IgM syndrome and immune deficiencies found in CD4 knockout mice; B-cells from such CD4- mice cannot undergo immunoglobulin class switching in response to T-cell dependent antigens (Monson et al., 2001). *In vitro* stimulation of APCs using various forms of CD40 agonists like membrane-associated CD40L, soluble CD40L, or anti-CD40 antibodies evokes distinct functional responses (Fanslow et al., 1994). Soluble CD40L is considered to be a pro-inflammatory cytokine because it has been shown to be involved in chronic inflammatory diseases like autoimmune disorders, arthritis, atherosclerosis, and even cancer (Laman et al., 1997; Cooke et al., 1999) . Elevated levels of soluble CD40L are also significantly associated with high risk for diabetes (Santilli et al., 2007), coronary and cerebrovascular atherosclerosis (Lobbes et al., 2006), and hypertensive and autoimmunity diseases (Desideri et al., 2007).

Agonistic anti-CD40 antibodies/recombinant CD40L have been successfully used as substitutes for T-cell help to regulate the activities of APCs both *in vitro* and *in vivo* in mammals and chickens (Bennett et al., 1998; Kothlow et al., 2008; Chen et al., 2010). Agonistic monoclonal antibodies (mAbs) against mouse CD40 have also been shown to directly mimic T-cell help *in vivo* in response to T-cell dependent / independent antigens in mice (Barr and Heath, 1999; French et al., 1999). Direct mimicking of T-cell help to B-cells by agonistic anti-CD40 mAbs may have therapeutic value in a T-cell deficiency syndrome like AIDS and hyper-IgM syndrome (Barr et al., 2003). Agonistic anti-CD40 mAbs can also confer a potent adjuvant effect when chemically conjugated with antigen, and the adjuvant effect can be attained against any physically attached antigen inducing protective immune responses against pathogens or tumors (Barr et al., 2003; Barr et al., 2006).

Agonistic monoclonal anti-CD40 antibody-based targeted vaccine

While there is great potential for novel vaccines based on recombinant proteins and synthetic peptides, such antigens often lack the immunogenicity of whole killed pathogens used in traditional vaccines. Novel vaccines require potent immunological adjuvants to render the antigens sufficiently immunogenic. Thus, the identification of immunological adjuvants with low reactogenicity and high potency to enhance humoral immune responses is quite necessary (Barr et al., 2003). Most immunologic adjuvants work through pattern recognition receptors, such as Toll-like receptors, on leukocytes by mimicking a danger signal to the immune system. These adjuvants induce release of cytokines, which often results in side effects at the injection site or systemic allergy (Rock et al., 2005). Arguably one of the most successful strategies to attain this end consists of attaching the antigen to an

antibody against a co-stimulatory cell surface receptor expressed by antigen presenting cells (APC), such as CD40 (Barr et al., 2006). A novel strategy of vaccine development using agonistic anti-CD40 antibody mAb, was reported to work directly on APCs with low reactogenicity but high immunogenicity. This strategy was able to enhance humoral immune responses 1000-fold compared with the use of an aluminum based adjuvant (Barr et al., 2006), and may avoid the inflammatory side effects induced by most adjuvants (Gendelman et al., 2005; Barr et al., 2006; Hamzah et al., 2008).

Unlike the activation pathway through pattern recognition receptors, Barr and co-workers reported that the adjuvant effect of agonistic anti-CD40 mAb is attributed to the CD40 binding domain and mediated by simple direct targeting and stimulating of B-cells, not through Fc receptor (Barr et al., 2003). Agonistic anti-CD40 mAb can directly mimic T-cell help to enhance humoral immune responses to T-cell dependent antigens; similar effects are also found when the same antigen is given with T-cell independent antigens and cytokines, such as IL-4, *in vivo* (Barr et al., 2003). Agonistic anti-CD40 antibodies not only target antigen delivery and activate B-cells, but also induce antibody class-switching. Murine and human naïve B-cells can be activated with anti-CD40 mAbs or CD40L (CD154) to undergo class switch recombination *in vitro* (Petersen et al., 2006). This is crucial, because IgA is readily transported across the intestinal mucosa and is endowed with effector properties that are critical for the local humoral immune response (Ravdin et al., 2006).

For T-cell independent antigens, administration of the anti-CD40 mAb with type III pneumococcal capsular polysaccharide induced the generation of strong, isotype-switched humoral immune responses, which are protective and extremely persistent (Diehl et al., 1999; French et al., 1999; Sotomayor et al., 1999; Ninomiya et al., 2002; Hatzifoti and Heath,

2007). This provides a good solution to overcome a major deficiency with polysaccharide based vaccines for infants and young children who fail to respond to many T-cell independent antigens. Activation through CD40 on B-cells by the administration of agonistic anti-CD40 mAb or recombinant CD40L in combination with cytokines leads to immunoglobulin class switching in a similar way as the stimulation of LPS plus cytokines (Diehl et al., 1999; French et al., 1999; Sotomayor et al., 1999; Ninomiya et al., 2002; Barr et al., 2003; Barr et al., 2005; Barr et al., 2006). The adjuvant effect of agonistic anti-CD40 mAb is T-cell independent, which is a definite advantage for the vaccination of patients with CD4 deficiencies, like those infected with HIV.

The induction of cell-mediated immunity has also been shown by synergistic activity with administration of agonistic anti-CD40 mAbs in mammals (Jabara et al., 1990; Schwabe et al., 1997; Khalil and Vonderheide, 2007). Antigen-specific CD8+ T-cells can be elicited from combination of stimulation with a Toll-like receptor (TLR) agonist and agonistic anti-CD40 mAb, and this combination optimizes the efficiency of a vaccine in eliciting both cell-mediated and humoral immunity (Ahonen et al., 2004; Barr et al., 2005).

Besides enhancing specific humoral immune responses, another concern of a vaccine is the long-term protection against specific immunogens. A potent enhancement of anamnestic responses against a second injection of antigen alone was reported (Barr et al., 2003), indicating a strong boosting of immunological memory. This formation of immunological memory fits the requirement for many vaccines.

Single-chain variable fragment (scFv)

However, large-scale of growing hybridoma for producing mAb is expensive and highly concentrated mAbs can only be produced in mouse ascites. Unfortunately, production of mAbs in mouse ascites has negative animal welfare implications, and several countries have legislation limiting antibody production in mouse ascites. These factors restrict the use of mAb based tests, vaccines, and therapeutics.

The scFv technology, cloning of a fusion protein of the variable domains of the heavy (V_H) and light chains (V_L) of immunoglobulin, is an alternative for classical monoclonal antibody preparation, which allows rapid but cheap production of functional proteins in substantial quantities. The smallest functional fragment of an antibody, which is composed of merely V_H and V_L, retains the binding site and specificity of the whole immunoglobulin molecule. The recombinant version of these fragments of variable regions, which lacks the constant region, are made into a more stable binding single chain fragment by covalently linking the two domains with a flexible polypeptide linker. The specificity is usually unaltered (Peterson and Adham, 2006). These molecules were created to facilitate phage display, where it is highly convenient to express the antigen-binding domain as a single peptide (Marks et al., 1991). As an alternative, scFv can also be created directly from subcloned V_H and V_L derived from a hybridoma instead of hyper-immune mouse splenocytes (Chaudhary et al., 1990).

V_H and V_L are usually joined together with a short linker peptide of 7 to 15 amino acids to form a monomer. The linker is rich in glycine for flexibility, as well as serine / threonine for solubility, and can either connect the N-terminus of V_H with the C-terminus of the V_L, or vice versa (Holliger et al., 1993). Shorter linkers (one / two amino acids) can also

lead to the formation of scFv trimmers (tribodies) and tetramer (tetrabodies) (Ravn et al., 2007). While the linker peptide was too short (five to seven amino acids), V_H and V_L cannot fold together by noncovalent binding. This scFv are forced to dimerize to form dimer known as diabody (Holliger et al., 1993). Diabodies are reported to have dissociation constant up to 40-fold lower than other corresponding scFv monomer (Holliger et al., 1993). The lower dissociation constant means diabody has much higher affinity to its corresponding antigen and may be a functional substitute of complete immunoglobulin (Le Gall et al., 1999). All of these different forms of scFv can be further modified to compose from different sets of V_H and V_L with specificity for more than a single antigen, in which case they are functional equivalence to bispecific antibodies (Muller and Kontermann, 2010).

A bispecific mAb (BsMAb) is a recombinant protein that is composed of two sets of V_H and VL from two different mAbs separately, and consequently can bind to two different types of antigen (Sarkar et al., 2012). The most widely used application of this BsMAb is in cancer immunotherapy, where BsMAbs are engineered to bind to cytotoxic T-cell (CD3+) and tumor cells simultaneously. In sum, tumor cells will be linked to few cytotoxic T-cells in the microenvironment, which will be destroyed by released cytotoxins perforin, granzymes, and granulysin (Lindhofer et al., 1995; Baeuerle and Reinhardt, 2009). The latest development of BsMabs is the bispecific tandem di-scFvs, known as bi-specific T-cell engagers (BiTEs) (Baeuerle and Reinhardt, 2009; Muller and Kontermann, 2010).

ScFvs diabodies have many applications like antigen-binding domains of artificial T cell receptors (Kolly et al., 2007), and sometimes can be functional substitute of classical antibodies in flow cytometry and immunohistochemistry (Gray et al., 2010). Unlike monoclonal antibodies, which are often produced in mammalian cell cultures, scFvs can be

in either prokaryotic or eukaryotic expression system (Peterson et al., 2006a). The scFv can then be modified for expression in a variety of systems, and for various applications, such as vaccine development and immunotherapy (Holliger et al., 1993). By fusing in-frame, the scFv to another molecule, a moiety can be achieved that is specific for a particular target with an enhanced function (Todorovska et al., 2001). Consequently, for highly specific targeting cells *in vivo*, the dose of therapeutic scFv diabody drug can be much lower than classical antibodies (Adams et al., 1998).

Adenovirus-mediated antigen delivery *in ovo*

Multiple experimental recombinant vaccines have been developed for chickens in many years, some of which have been reported to efficiently protect chickens from diseases, especially from avian influenza (Toro et al., 2007). The distinct advantage of vectored vaccines, especially live replication-defective vectored adenovirus, is the ability to introduce highly specific and well-defined antigens that can be the focus of specific immune reactivity without the risk of generating virulent revertants (Breedlove et al., 2011). Recombinant adenoviruses have tremendous potential in both research and therapeutic applications (Marukawa et al., 2012). There are numerous advantages they provide when introducing genetic material into host cells and the permissive host cell range is very wide (Xin et al., 2007; Naskalska et al., 2009). After entering cells, the virus remains epichromosomal – the adenoviral genome does not integrate into the host chromosome so does not activate or inactivate host genes -. Recently, recombinant adenoviruses have been used to deliver RNAi into cells (Nishioka et al., 2012), and are especially useful for gene transfer and protein expression in cell lines that have low transfection efficiency with liposome (Byrnes et al.,

1995). The replicative and non-replicative adenovirus has been used to infect many cell types from mammals and chickens for high expression of the recombinant protein (Fisher and Watanabe, 1996; Hermening et al., 2004).

Non-replicative adenovirus-vectored antigen delivery systems in chickens provide a means whereby the problems associated with modified live viruses and inactivated viruses can be overcome (Avakian et al., 2007). Recombinant replication-defective human adenoviruses (Ad), such as adenovirus type 5 (Ad5) combine a number of very promising properties. First, despite its human origin, vectored adenovirus is able to target foreign genes in a wide spectrum of animal cells with maximum efficacy, even those in which Ad5 cannot replicate (Ali et al., 1994). Second, the viral DNA is transiently maintained and expressed for several weeks to months; witch produces long lasting immunity against the foreign protein (Alemany et al., 2000).

Transduction of chicken cells *in vitro* and *in vivo* by replication-defective, thus non-spreading, adenovirus type 5 (Ad5) was reported for the first time in 1995 (Adam et al., 1995). Human adenoviral vectors potentially transduce chicken cells through binding of the adenovirus fiber to the coxsackie virus and adenovirus receptor on the surface of chicken cells (Tan et al., 2001). More recently, chickens immunized with a single subcutaneous injection of an adenovirus-vectored avian influenza H5 antigen were 100% protected from an intranasal challenge with VN/1203/04, which proved lethal to all control-vaccinated chickens within 2 days (Gao et al., 2006). In addition, at least one of the human adenovirus components, hexon, is highly immunogenic and confers adjuvant activity to exogenous antigens (Molinier-Frenkel et al., 2002).

Protective humoral response against avian influenza was reported by *in ovo* vaccination with a non-replicating vectored human adenovirus (Toro et al., 2007). Delivery to the amniotic fluid of the embryo is highly effective because during E17-19, the embryo imbibes the amniotic fluid and viral particles are rapidly found in the respiratory tract and in the digestive system (Jochemsen and Jeurissen, 2002; Kapczynski et al., 2003), resulting in vaccine delivery to the mucosa-associated lymphoid tissue (MALT) as well as the bursa of Fabricius, the avian primary B-cell organ. Exposure to antigen drives the maturation of avian MALT (Jeurissen et al., 1989; Reese et al., 2006) and suggests that *in ovo* immunization may accelerate the maturation of embryonic bronchus-associated lymphoid tissue (BALT) (Rautenschlein and Haase, 2005).

In ovo vaccination plays an important role in protecting chickens from several diseases (Josefsberg and Buckland, 2012). This injection system vaccinates up to 70,000 chicken eggs per hour in a more uniform and precise manner than post-hatch mass vaccination methods (Avakian et al., 2007). Today, approximately 90% of broiler chickens grown in the USA are vaccinated *in ovo* 2-3 days prior to hatch at the time when eggs are transferred from the incubator to the hatcher (Avakian et al., 2007). Commercial *in ovo* vaccination using modified live viruses is common practice for Marek's disease (MD), infectious bursal disease (IBD) and fowl pox (FP). In other cases, however, *in ovo* compatibility has been problematic, as in the case of the modified live viruses for Newcastle Disease (ND) and infectious bronchitis (IB), because of acute pathogenic effects on the embryo (Sharma et al., 2002).

In ovo delivery of adenovirus-vectored vaccines makes it possible to mass-administer a wide variety of pathogen-derived antigens, delivers high potency in a single-dose regimen, does not interfere with epidemiological surveys of natural infections, and is not hindered by

pre-existing (maternal) immunity in chickens to human adenoviruses – at least at the experimental level. Finally, adenovirus-vectored vaccines can be generated rapidly and mass-produced cheaply (Avakian et al., 2007).

Since broilers are reared with a short life span (≤ 2 months), *in ovo* vaccination with replication-defective vectored adenovirus should be able to protect the great majority of chickens against Cpα and significantly reduce food borne disease in human. Introduction of this new class of *in ovo* vaccination may thus provide a simple and safe means for mass immunization of poultry in a wide variety of disease settings.

CHAPTER III

PRODUCTION AND CHARACTERIZATION OF AGONISTIC MONOCLONAL ANTIBODIES AGAINST CHICKEN CD40

Introduction

As a consequence of phylogenetic separation of chickens from the reptile ancestor of mammals about 300 million years ago, the divergence of immune system of chicken from that of mammal represents an important milestone in the evolution of immune system in vertebrates (Furlong, 2005). Antibody-secreting B-cells are present in most jawed vertebrates, including chickens and mammals (Flajnik, 2002), and the diversity of B-cell receptor (BCR) is generated in different fashions among vertebrate species (Richards and Nelson, 2000). The huge diversity of BCRs of chickens and mammals are generally generated by V(D)J recombination and somatic hypermutation on immunoglobulin genes (Du Pasquier, 1993). However, V(D)J recombination only produces very limited diversity, somatic hypermutation / gene conversion contribute mostly the wide spectrum diversity of BCRs in chickens (Thompson and Neiman, 1987). The gut-associated lymphoid tissues, such as bursa of Fabricius, provide another specialized environment for extra diversification stage of B-cells development in chickens (Withers et al., 2006). However, very little information regarding molecular signals for controlling development, survival, and apoptosis of chicken B-cells is available (Kothlow et al., 2010).

In mammals, tumor necrosis factors (TNFs) play important roles in the development and regulation of the immune system, especially for B-cells (Williamson et al., 1983; Watts, 2005). The function of each TNF is essentially determined by its interaction with receptor

expressed on B-cells, which belongs to tumor necrosis factor receptor (TNFR) superfamily (Locksley et al., 2001). The TNFR, also called death receptor, is a trimetric cytokine receptor for TNF. Because TNF is often used to describe TNF-α, TNFR is often used to describe the receptors that bind only to TNF-α, also known as CD120. The TNFR also cooperates with adaptor proteins like tumor necrosis factor-associated factors (TRAFs) to determine the outcome while it was bound by corresponding TNF (Baker and Reddy, 1998). Both lymphotoxin-α and TNF-α were found in the chicken, several members of the chickens TNF/TNFR family have also been identified, including Fas/FasL, Ox40L, Trail/TrailL, CD30/CD30L, and Rank/RankL (Burgess et al., 2004; Hong et al., 2006).

However, several TNFRs can react to more than one TNF (Locksley et al., 2001; Hehlgans and Pfeffer, 2005). TNF family members share a low degree of sequence similarity, with the majority of conserved residues involved in maintaining the structure of the C-terminal domain of TNF (Idriss and Naismith, 2000). The majority of TNF are type II transmembrane proteins, and reminding are mostly in secretory fashion, which may be cleaved from the membrane by proteases and released as soluble proteins (Tan et al., 1997).

Recent progress has been made by cloning and expressing chicken CD40L (CD154) and BAFF, which belongs to TNF family members and both are essential for differentiation and development of chicken B-cells. Like mammal BAFF, chicken BAFF was shown to function as survival factor for mature peripheral B-cells (Schneider et al., 2004). CD40L has several essential functions in the regulation of adaptive immunity in mammals, as clearly was evidenced by the severe consequences of inactivating mutations in X-linked hyper IgM syndrome (Aruffo et al., 1993; Pearson et al., 2001). CD40L not only provides signals for the maturation of dendritic cells, which play critical role in the initiation of T-cell response

(Moodycliffe et al., 2000), but is also required for the formation of germinal centers, affinity maturation, and generation of class-switched memory B-cells in T-cell dependent humoral responses (Foy et al., 1994). Compared to the broad distribution of CD40 on cells and tissues, CD154 was initially reported to be expressed by activated T-cells (Armitage et al., 1992). The chicken CD154 was found to be expressed on both activated CD4 and CD8 T-cells, and larger proportion of chicken CD8 T-cells than mammalian CD8 T-cells express this molecule upon activation (Tregaskes et al., 2005).

CD40, an integral membrane glycoprotein of the TNF-receptor superfamily, is expressed mainly on professional antigen-presenting cells (APCs), including B-cells, macrophages, and dendritic cells (van Kooten and Banchereau, 1997; van Kooten and Banchereau, 2000). As well as APcs, a variety of non-immune cells also express CD40, the cognate receptor for CD154, and can respond to a CD154 stimulus by secreting proinflammatory cytokines (Dechanet et al., 1997). However, high levels of CD40 can also be detected on a wide range of human cancer cells of bladder, breast, and ovary (Sabel et al., 2000; Hill et al., 2005). The natural ligand for CD40 is CD154 (CD40L), which is transiently expressed on activated $CD4^+$ T-cells (Clark and Ledbetter, 1986; Armitage et al., 1992). The engagement of CD154 to the CD40 receptor on APCs provides the critical signal required for optimal APC activation, which results in up-regulation of MHC and CD40 molecules, expression of CD80/86, and secretion of cytokines (Grewal and Flavell, 1996; Noelle, 1996). These steps are critical for optimal priming and expansion of antigen-specific effector and memory T-cells, B-cell response, and immunoglobulin class switching (van Kooten and Banchereau, 1997; Gordon and Pound, 2000).

Agonistic anti-CD40 antibodies/recombinant CD40L have been successfully used as substitutes for $CD4^+$ T-cell help to regulate the activities of APCs both *in vitro* and *in vivo* in mammals and chickens (Bennett et al., 1998; Ridge et al., 1998; Tregaskes et al., 2005; Kothlow et al., 2008). Agonistic monoclonal antibodies (mAbs) against mouse CD40 have also been shown to directly mimic $CD4^+$ T-cell help *in vivo* in response to T-cell dependent antigens (Banchereau et al., 1994; Dullforce et al., 1998; Barr and Heath, 1999; French et al., 1999; Garcia de Vinuesa et al., 1999). Such mAbs can also confer a potent adjuvant effect when physically conjugated with antigen (Diehl et al., 1999; French et al., 1999; Sotomayor et al., 1999; Ninomiya et al., 2002; Barr et al., 2003; Barr et al., 2005; Barr et al., 2006), inducing protective immune responses against pathogens or tumors (Diehl et al., 1999; French et al., 1999; Sotomayor et al., 1999; Ninomiya et al., 2002; Hatzifoti and Heath, 2007).

In contrast to the extensive physico-biochemical and functional characterization of human and murine CD40 by use of agonistic anti-CD40 mAbs, relatively few reports are available on this topic in the chicken, except for two recent seminal publications by Tregaskes and co-workers (Tregaskes et al., 2005; Kothlow et al., 2008), who identified chicken CD40 (chCD40) and characterized the chCD40/chCD40L ligation using recombinant chCD40L. In this study, we describe for the first time the development and characterization of an agonistic anti-chCD40 mAb, 2C5, which, like analogous agonistic anti-CD40 mAbs against mammalian CD40, is functionally active when specifically bound to CD40 on chicken APCs, causing downstream immunological effects. MAb 2C5 stimulated significant nitric oxide (NO) synthesis in chicken HD11 macrophages and dramatically stimulated the proliferation of serum-starved chicken DT40 B-cells.

Materials and methods

Cell cultures

Cell cultures were maintained at 37°C in an atmosphere of 5% CO_2. Chicken HD11 macrophages (Crippen et al., 2003) were grown in DMEM medium (Mediatech, Manassas, VA) supplemented with 8% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 5% chicken serum (Sigma, St. Louis, MO). Chicken DT40 B-cells (Winding and Berchtold, 2001) were cultured in DMEM medium supplemented with 10% fetal bovine serum, 1% chicken serum, 10% tryptose phosphate broth and 50μM β-mercaptoethanol. Chicken primary B-cells and primary macrophages were prepared according to published protocols with minor modifications (Endsley et al., 2009). Briefly, the spleen was surgically removed from a laying hen and rinsed in ice-cold serum-free RPMI 1640 medium (Sigma) containing 100 IU/mL of penicillin/streptomycin. The tissue was then mechanically disrupted using a surgical blade and a syringe plunger, and splenocytes were obtained by passing the preparation through a 70μm cell strainer (BD Falcon, San Jose, CA), and resuspended in RPMI 1640 medium supplemented with 8% fetal bovine serum, 5% chicken serum, and 25 nM phorbol-12-myristate-13-acetate (Cell Signaling Technology, Beverly, MA). Chicken primary B-cells were then isolated from splenocytes by positive selection using mouse anti-chicken Bu-1 mAb (SouthernBiotech, Birmingham, AL) as follows: after incubating splenocytes with Bu-1 mAb for 30 minutes at 4°C, splenocytes were washed three times with ice-cold sorting buffer [0.5% bovine serum albumin (BSA), 2mM ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline (PBS), pH 7.4], incubated with goat anti-mouse immunoglobulin microbeads (Miltenyi Biotech, Auburn, CA) for 30 minutes at 4°C, and positively selected using the AutoMACS automated magnetic cell sorter (Miltenyi Biotech).

Chicken primary macrophages were derived by culturing adherent monocytes from splenocytes in RPMI medium containing 8% fetal bovine serum, 5% chicken serum, and 10μg/mL bovine insulin in a 6-well cell culture plate (Nunc, Rochester, NY) in an atmosphere of 5% CO_2 at 37°C for 13 days. Adherent cells were collected using Accutase (Sigma) and isolated by positive selection. Briefly, cells were incubated with mouse anti-chicken MHC-II mAb (SouthernBiotech) for 30 minutes at 4°C and then washed three times in ice-cold sorting buffer, incubated with goat anti-mouse immunoglobulin microbeads for 30 minutes, and positively selected using the AutoMACS automated magnetic cell sorter.

Generation of recombinant extracellular domain of chicken CD40 (chCD40$_{ED}$)

Total RNA was isolated from chicken spleen using Trizol® (Invitrogen, Carlsbad, CA) and used for cDNA synthesis using Superscript II reverse transcriptase (Invitrogen). The sequence encoding chCD40$_{ED}$ was then amplified by nested PCR using Accuprime *Pfx* DNA polymerase (Invitrogen). Primers (inside and outside) used in this nested PCR are shown in Table1. The PCR product was gel-purified, ligated into the pcDNA 3.1/V5-HIS-TOPO vector (Invitrogen), and 5μL of the ligation reaction was used to transform Top 10 *E. coli* cells (Invitrogen). Plasmid minipreps with cDNAs encoding chCD40$_{ED}$ were sequenced, and the cDNA encoding chCD40$_{ED}$ was amplified by overlapping extension PCR using primers shown in Table 1. The forward primers (CD6chCD40 Fwd-1, CD6 Eco RV Fwd-2, and CD7 Fwd) introduced an *Eco* RV restriction site (in bold) and the CD5 secretory signal sequence (underlined) at the 5' terminus (Table 1). The reverse primer (cCD40 Flag Rev) introduced a *Bam* H1 restriction site (in bold) and the FLAG-tag sequence (underlined) at the 3'-terminus (Table 1). This construct was then sub-cloned into the eukaryotic expression vector pcDNA5

(Invitrogen) and the gene was confirmed by DNA sequencing. Recombinant chCD40$_{ED}$ (Fig. 1) was expressed as a FLAG-tagged protein (Fig. 2) in HEK 293 Free-Style cells (Invitrogen) and affinity purified using anti-FLAG M2-agarose affinity chromatography (Sigma) (Hope et al., 2005). This chCD40$_{ED}$ was used for immunization of Balb/c mice for the production of monoclonal antibodies.

Table 1. Primer sequences used in cloning of extracellular domain of chicken CD40

Primer Name	Direction	Primer sequence
Outside	Forward	5' – GGG TCG GAT CGG ATC GGA TGG G – 3'
Outside	Reverse	5' – GCT GCT CCC GTC GGA GGC – 3'
Inside	Forward	5' – CAC CAT GGG GCG GCT CGG GCT GCT – 3'
Inside	Reverse	5' – CTG CTC CTG CTC GGC GAT GCG GCT – 3'
CD6cCD40 Fwd-1	Forward	5' – ACC TTG TAC CTG CTG GGG ATG CTG GTC GCT TCC TGC CTC GGA CTG CAG ATG GTG AAT TGC TCT GAC AAG CAG – 3'
CD7 Fwd	Forward	5' – ATA GAT ATC GCC AGC CAT GGC CAT GGG GTC TCT G – 3'
CD6 EcoRV Fwd-2	Forward	5' – ATA **GAT ATC** ACC ATG CCC ATG GGG TCT CTG CAA CCG CTG GCC ACC TTG TAC CTG CTG GGG ATG CTG – 3'
cCD40 Flag Rev	Reverse	5' – ATA **GGA TCC** TTA CTT ATC GTC ATC GTC CTT GTA GTC TGA CAG CGA TGA GCG CCG GCT – 3'

```
1       10         20         30         40         50         60
MPMGSLQPLA TLYLLGMLVA SCLGLQVNCS DKQYEHKGRC CNRCQPGKKL ASECNDTEDS

        70         80         90        100        110        120
VCTPCENGQY QHSWTKERHC TPHEICEDNA GLIVKRHGNA THNTVCQCRA GMHCSDASCQ

       130        140        150        160        170        180
TCVENEPCKQ GFGFVAAMAE ARMTSPCEPC AEGTFSNVSS KTEPCHFWTS CEEKGLVVKV

       190        200
KGTNTSDVIC ESSRRSSLSD YKDDDDK
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Fig. 1. Amino acid sequence of CD5-chCD40$_{ED}$-FLAG chimeric protein. Recombinant cDNA technology was used to produce chCD40$_{ED}$ (amino acids 27-199) linked in-frame to the sequence encoding the CD5 secretory signal at the N-terminus (amino acids 1-26, bolded), and the FLAG-tag sequence (amino acids 200-207, bolded italics) at the C-terminus.

Monoclonal antibody production

Monoclonal antibodies against chCD40 were produced according to previously published protocols used routinely (Mast et al., 1998). Briefly, four female BalB/c mice were injected four times subcutaneously with 50µg chCD40$_{ED}$ in RIBI adjuvant [1mg Lipid A, monophosphorylated from *E. coli* F583 (Sigma), 1mg Trehalose 6, 6′-dimycolate from *Mycobacterium tuberculosis* (Sigma), 0.4% (v/v) Tween-80 (Sigma), 4.5% (v/v) squalene in 2mL MQ-water]. Mice with the best immune response against chCD40$_{ED}$ were activated by intraperitoneal injection of 50µg chCD40$_{ED}$ without adjuvant at three and five days prior to splenocyte harvest. On the day of fusion, a splenocyte suspension was prepared and fused with Sp2/0 myeloma cells (ATCC, Manassas, VA) by electrofusion using the Electro Cell Manipulator® ECM 2001 (BTX, Holliston, MA). Hybridomas were plated in 96-well cell culture plates (Nunc) with addition of cytokines (Berghman et al., 1992) to sustain single cell growth, and HAT selection was applied for seven days. Primary screening was performed by ELISA on day 14 post-fusion using ELISA plates coated with chCD40$_{ED}$. Positive clones (27 clones) from the primary screening were further tested by immunocytochemistry (ICC) on chicken DT40 B-cells and chicken HD11 macrophages. Briefly, cells were washed three

times with ice-cold PBS, pH 7.4, followed by fixation on poly-L-lysine coated slides using 4% (w/v) paraformaldehyde in PBS, pH 7.4, for 10 minutes. Cells were blocked for one hour in 5% (w/v) BSA (Sigma) in TBST [25 mM Tris-HCl, pH 7.6, 0.15 M NaCl, 0.1% Triton X-100 (v/v)]. Each slide was then incubated with 3mL hybridoma supernatant (27 hybridoma supernatants were tested separately) at room temperature for two hours, followed by incubation with FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 500-fold in TBST at room temperature for one hour. Specific staining on cells was visualized using a Zeiss Axioplan Microscope (Zeiss, Hamburg, Germany) and analyzed using Axio imager software (Zeiss). Twenty-four clones were thus identified and agonistic clones were further selected from those ICC positive clones using functional assays as described below.

Selection of agonistic anti-chCD40 monoclonal antibodies

Agonistic anti-chCD40 mAbs were selected based on (1) induction of NO synthesis in chicken HD11 macrophages *in vitro*, and (2) enhancement of proliferation in serum-starved chicken DT40 B-cells *in vitro*, as described below. Out of the 24 clones that stained chicken DT40 B-cells and HD11 macrophages, five agonistic anti-chCD40 hybridomas were cloned by limiting dilution, and the most promising clone 2C5 (IgG1) was sent to Antagene Inc (Sunnyvale, CA) for ascites production. The isotypes of five mAbs were determined using the ELISA-based isotyping kit (Zymed, South San Francisco, CA).

Purification of anti-chCD40 mAbs

Anti-chCD40 mAbs were purified from hybridoma supernatants or from ascites by Protein A Sepharose affinity chromatography. Briefly, one volume of supernatant of hybridoma culture or ascites fluid was diluted in eight volumes of application buffer [1.5M glycine, 3M NaCl in MQ-water, pH 8.9], mixed with immobilized Protein A (Thermo Scientific, Rockford, IL) for one hour on an end-over-end mixer. After rinsing with application buffer, the purified antibody was eluted with 50mM citric acid, pH 3.5 and dialyzed against PBS, pH 7.4.

Detection of nitric oxide (NO) by the Griess assay

To detect the concentration of NO synthesized by chicken HD11 macrophages stimulated by agonistic anti-chCD40 antibodies, chicken HD11 macrophages ($1x10^6$) were cultured in a 24-well cell culture plate in an atmosphere of 5% CO_2 at 37^oC for 12 hours; the plates were then rinsed three times with medium to remove unattached cells, followed by incubation with either anti-chCD40 hybridoma supernatants (3mL) or purified anti-chCD40 mAbs (1µg/mL) for 18 hours. A mouse IgG1 (1µg/mL) was used as a negative control. Nitrite concentration, a measure of NO synthesis, was assayed in 50µL of culture supernatant using the Griess reagent; 100µL sulfanilamide solution [1% (w/v) sulfanilamide (Sigma) in 2.5% (v/v) phosphoric acid in MQ-water] was added to the supernatants, followed by addition of 100µL naphthylethylenediamine dihydrochloride solution [0.1% (w/v) naphthylethylenediamine dihydrochloride (Sigma) in 2.5% (v/v) phosphoric acid in MQ-water) (Bingaman et al., 2000; Shoda et al., 2000; Shoda et al., 2001a; Shoda et al., 2001b). Absorbance at 550nm was compared to that of a freshly prepared $NaNO_2$ standard curve

(ranging from 0 to 160μM), and was used to calculate mean micromolar concentration of nitrite in triplicate cultures ± SD. Accumulation of nitrite was analyzed for statistical significance using the t-test. Out of the 24 anti-chCD40 hybridomas that were screened, 21 clones were found to stimulate NO production in chicken HD11 macrophages. The five best clones (designated as 2C5, C6, 1B5, C19, and 1G1) were then cloned by limiting dilution, purified by protein A Sepharose affinity chromatography, and used at four concentrations (0.3, 1, 3, or 10μg/mL) to assess dose-dependent NO synthesis by chicken HD11 macrophages.

B-cell proliferation assay

To assess the capacity of the selected agonistic anti-chCD40 mAbs to stimulate proliferation of chicken DT40 B-cells, the minimal concentration of fetal bovine and chicken serum necessary for survival in chicken DT40 B-cell medium was first determined. Chicken DT40 B-cells were cultured in DMEM medium supplemented with 0.156% (v/v) fetal bovine serum, 0.0156% (v/v) chicken serum, 10% (v/v) tryptose phosphate broth and 50μM β-mercaptoethanol. Each well of a 24-well cell culture plate was seeded with chicken DT40 B-cells ($5x10^5$) in an atmosphere of 5% CO_2 at 37°C, with addition of either one of the five different purified agonistic anti-chCD40 mAbs at 1μg/mL, or mouse IgG1 (1μg/mL) as a negative control. Cell densities (mean cell number counts per 24 hours in triplicate cultures ± SD) were determined at 24, 48, and 72 hours after the addition of the mAbs. Cellular proliferation (as assessed by the trypan blue exclusion test) was analyzed for statistical significance by the Mann-Whitney test. Dose-dependent B-cell proliferation was further tested at four concentrations: 0.3, 1, 3, or 10μg/mL.

Flow cytometric assay

Fc receptors on cells (DT40 B-cells, HD11 macrophages, primary chicken B-cells, and primary chicken macrophages) were blocked with purified mouse immunoglobulin at 200µg/ml for 30 minutes at 4^{o}C prior to staining, and Zenon labeling technology (Invitrogen) was used for labeling 2C5 (or mouse IgG1) with R-Phycoerythrin per the manufacturer's instructions. Briefly, cells (1 x 10^5) were incubated with various dilutions of R-Phycoerythrin-labeled 2C5 for 30 minutes at 4^{o}C, followed by fixation with 2% formaldehyde in PBS, pH 7.4, Flow cytometric analysis was performed using FACSCalibur system (BD), and data were analyzed using FlowJo version 8.8.4 software (Tree Star, Inc., Ashland, OR).

Immunoprecipitation

Chicken DT40 B-cells or chicken HD11 macrophages ($1x10^8$) were washed three times with ice-cold PBS and then incubated in 2mL of ice-cold lysis buffer [1% Triton X-100 (v/v), 150mM NaCl, 50mM Tris base, pH 8.0], supplemented with Halt^{TM} protease and phosphatase inhibitor cocktail (Thermo Scientific) for 30 minutes on ice. After centrifugation at 16,000 x g for 15 minutes, the supernatant was collected and pre-cleared by incubation with 100µL protein G agarose (Thermo Scientific) and 20µg purified mouse IgG (Jackson ImmunoResearch) for one hour to reduce nonspecific binding. Mouse IgG and protein G agarose were removed by centrifugation at 10,000 x g for five minutes. A pool of agonistic anti-chCD40 mAbs was prepared by mixing 4µg of each of the five purified agonistic mAbs (2C5, C6, 1B5, C19, and 1G1). Lysates were immunoprecipitated with this mAb pool and 100µL protein G agarose (Thermo Scientific) for two hours at 4^{o}C on an end-over-end mixer.

Immune complexes were collected by centrifugation, washed three times with ice-cold lysis buffer, and then boiled for 10 minutes at 95^{o}C in 50µL 1X Laemmli sample buffer containing 50mM fresh dithiothreitol (Bio-Rad, Hercules, CA) and 5% 2-mercaptoethanol. Iodoacetamide was then added to a final concentration of 25mM and immune complexes were separated by SDS-PAGE and identified by Western blotting.

Western blotting

Samples were separated by SDS-PAGE using a 10% Precise^{TM} protein gel (Thermo Scientific) under reducing conditions, and then transferred to a PVDF membrane (Bio-Rad) for 90 minutes at 40V in transfer buffer [25mM Tris, 192mM glycine in 800mL Milli-Q water and 200mL methanol]. Twenty µg reduced mouse IgG1 and 5µg BSA were used as the negative control. The membrane was blocked overnight in 5% skimmed milk (BD) in TBST [TBS containing 0.1% Tween-20 (v/v)], and then incubated with polyclonal mouse anti-chCD40 antibody diluted at 1:5000 in TBST containing 5% skimmed milk at room temperature for two hours. This was followed by incubation with the TrueBlot system (eBiosciences, San Diego, CA) in TBST containing 5% (w/v) skimmed milk at room temperature for one hour. This system includes a horseradish peroxidase (HRP)-conjugated antibody that is specific for native mouse IgG, thus eliminating the interference of IgG present in the purified immune complexes. The resulting bands on the membrane were developed using ECL Plus developer system (GE Healthcare, Foster City, CA), visualized using a ChemiDoc^{TM} XRS system (Bio-Rad), and analyzed using Quantity One imaging software (Bio-Rad).

Results and discussion

The CD40/CD40L signaling pathway is crucial in humoral and cell-mediated immunity in mammals and chickens. The primary structures of chCD40 and chCD40L were recently identified, and chCD40-chCD40L interaction was shown to induce NO synthesis in the chicken HD11 macrophage cell line and to support the proliferation of splenic B-cells (Tregaskes et al., 2005; Kothlow et al., 2008). To investigate the distribution and the biological function of chCD40, a novel agonistic anti-chCD40 mAb 2C5 was produced and characterized in this study.

To obtain recombinant $chCD40_{ED}$, a DNA construct encoding the extracellular domain of chicken CD40 linked in-frame to the sequence encoding the CD5 secretory signal sequence at the 5' terminus, and the sequence encoding the FLAG-tag at the 3' terminus, was generated in the eukaryotic expression vector pcDNA5. Protein expressed by the resultant construct, designated as $chCD40_{ED}$-FLAG, was verified in HEK 293A cells by immunocytochemistry (Fig. 2), whereas HEK 293 Free-Style cells (Invitrogen) were used to generate large amounts of recombinant FLAG-tagged $chCD40_{ED}$ that was affinity purified using anti-FLAG M2-agarose gel (Sigma). This protein preparation was used for immunization of Balb/c mice for the production of monoclonal antibodies.

A large number of hybridomas were obtained two weeks following electrofusion of splenocytes from hyper-immunized mice with Sp2/0 myeloma cells. Antibodies recognizing $chCD40_{ED}$ were secreted by 27 different hybridomas as identified by ELISA. Twenty four out of these 27 hybridomas secreted antibodies that also bound to native CD40 on the surface of chicken DT40 B-cells and HD11 macrophages, as determined by ICC (Fig. 3). No positive staining was observed in the negative control (mouse IgG1).

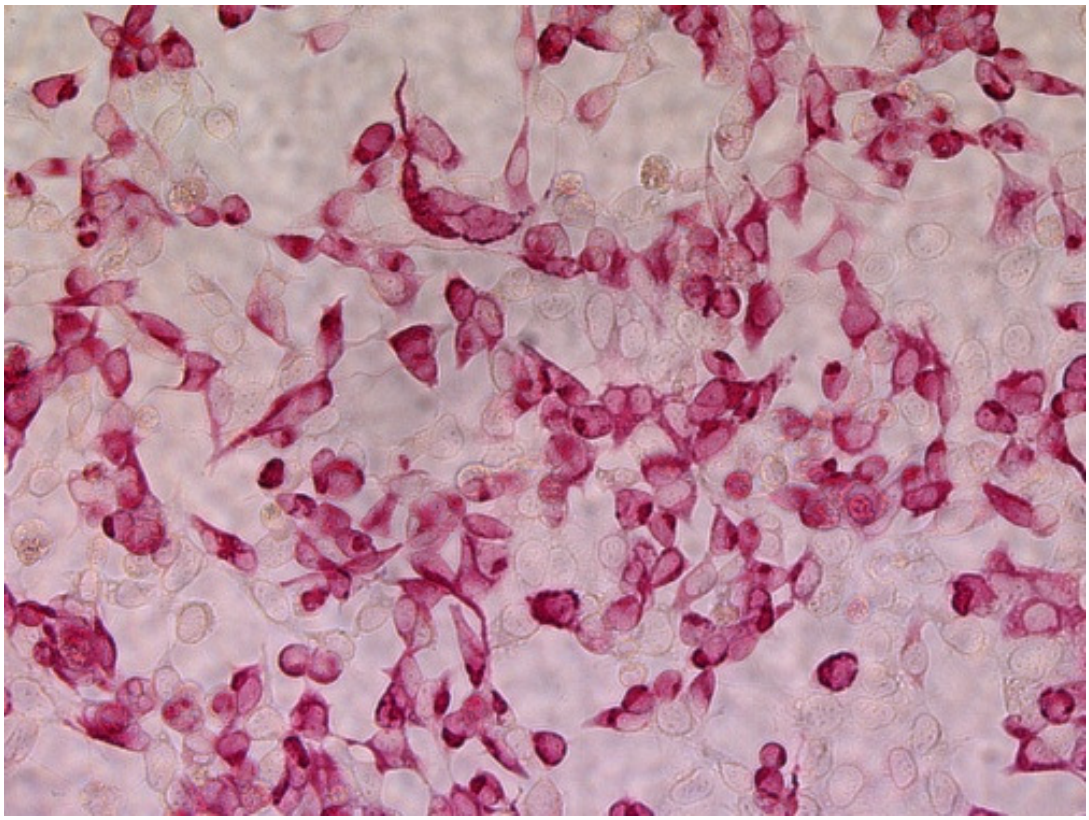

Fig. 2. Expression of chCD40$_{ED}$-FLAG in HEK 293A cells transfected with pcDNA5-(CD5-chCD40$_{ED}$-FLAG). 48 hours post transfection, expression of chCD40$_{ED}$-FLAG in transfected HEK 293A cells were visualized by red alkaline phosphatase (AP) staining (AP conjugated anti-FLAG mAb).

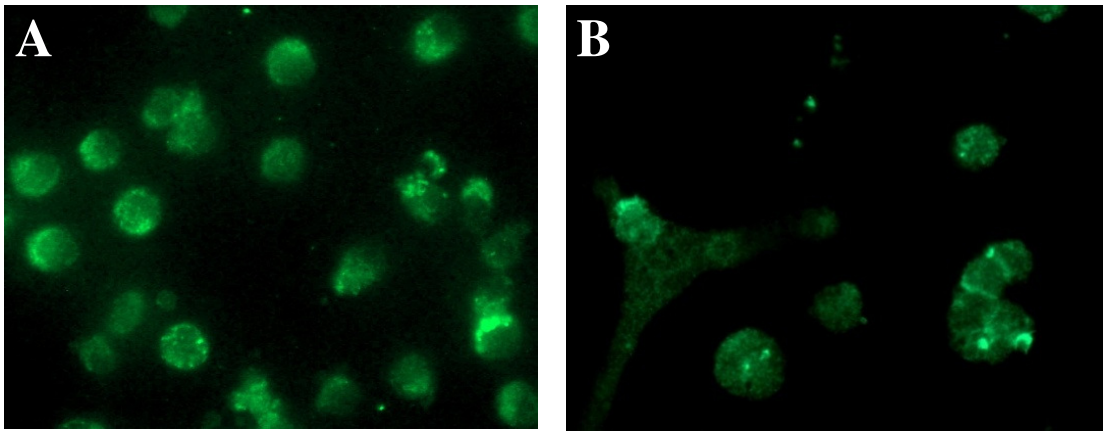

Fig. 3. Immunocytochemical detection of CD40 expressed on chicken DT40 B-cells (A) and chicken HD11 macrophages (B) stained with undiluted culture supernatant of anti-chCD40 hybridoma 2C5. Cells were fixed on poly-L-lysine coated slides using 4% paraformaldehyde in PBS, pH 7.4. FITC-conjugated goat anti-mouse IgG was used for detection.

In order to characterize 2C5 and facilitate its further use, the class and subclass identities of 2C5 were determined. Wells with the highest response [highest A(450)] indicate

isotype and light chain composition. 2C5 clearly have positive responses in IgG1 (heavy-chain identification) and κ (light-chain identification) (Fig. 4).

The binding of 2C5 to CD40 on the cell surface of chicken cells was confirmed *in vivo* by flow cytometry on chicken DT40 and HD11 cells (Fig. 5A and 5B), and with primary B-cells and macrophages (Fig. 5C and 5D). Pre-immune mouse IgG1 was used as the negative control. Primary chicken B-cells that had been selected with mouse anti-chicken Bu-1 mAb expressed high levels of CD40. Primary chicken macrophages differentiated from attached spleen monocytes were purified by mouse anti-chicken MHC-II mAb affinity chromatography (Fig. 6).

First, specific binding of 2C5 to CD40 on chicken APCs was demonstrated by immunocytochemistry. CD40 expression on chicken DT40 B-cells, HD11 macrophages, primary macrophages, and primary B-cells were confirmed by flow cytometry, indicating that 2C5 can be used for detailed screening of the expression level and distribution of chCD40 on various chicken cells and tissues. This expression profile confirms what was reported in studies that used recombinant chCD40L instead of an immunochemical reagent (Tregaskes et al., 2005).

Flow cytometric analysis showed that primary macrophages did express CD40, but at lower levels than primary B-cells (Fig. 5C and 5D). Specific binding of anti-chCD40 mAb to chCD40 was also further confirmed *in vitro* by immunoprecipitation (Fig. 7). From cellular extracts of both chicken DT40 B-cells and HD11 macrophages, a pool of agonistic anti-chCD40 mAbs precipitated molecules with apparent molecular weights of 55 and 32kDa.

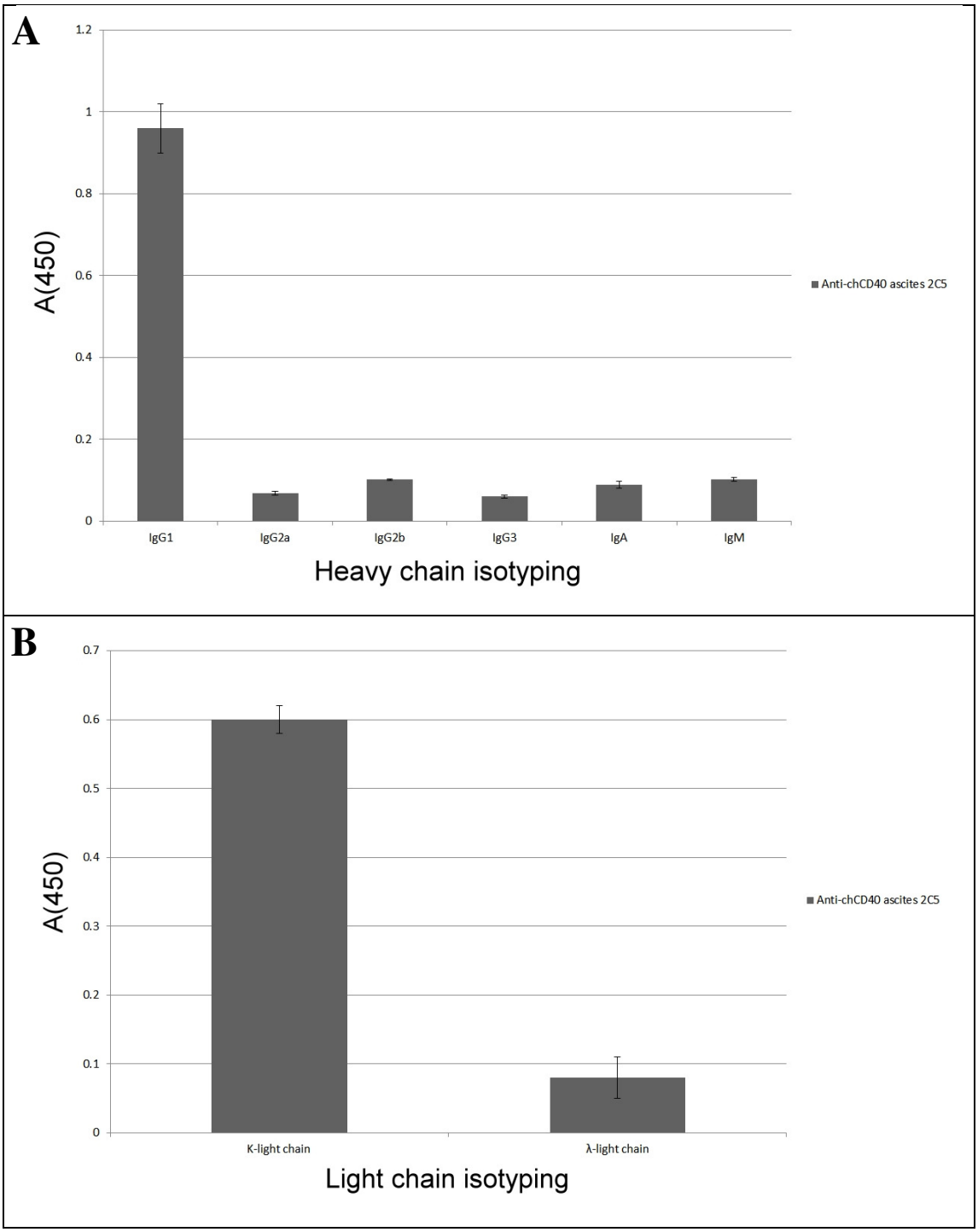

Fig. 4. Immunoglobulin isotyping on both heavy chain (A) and light chain (B) of agonistic anti-chCD40 mAb 2C5. ELISA plates were coated with goat anti-mouse immunoglobulin antibody (5μg/mL) and then incubated with agonistic anti-chCD40 2C5 ascites diluted in PBS (1:1000) followed by isotype antibodies from rabbit. Data are average values from three samples and are representative of three independent experiments. (A) Reactivity of heavy chain isotype antibodies to 2C5. Significant IgG_1 response to 2C5 was detected. (B) Reactivity of light chain isotype antibodies to 2C5. Significant κ response to 2C5 was detected. Error bars are calculated based on independent experiments.

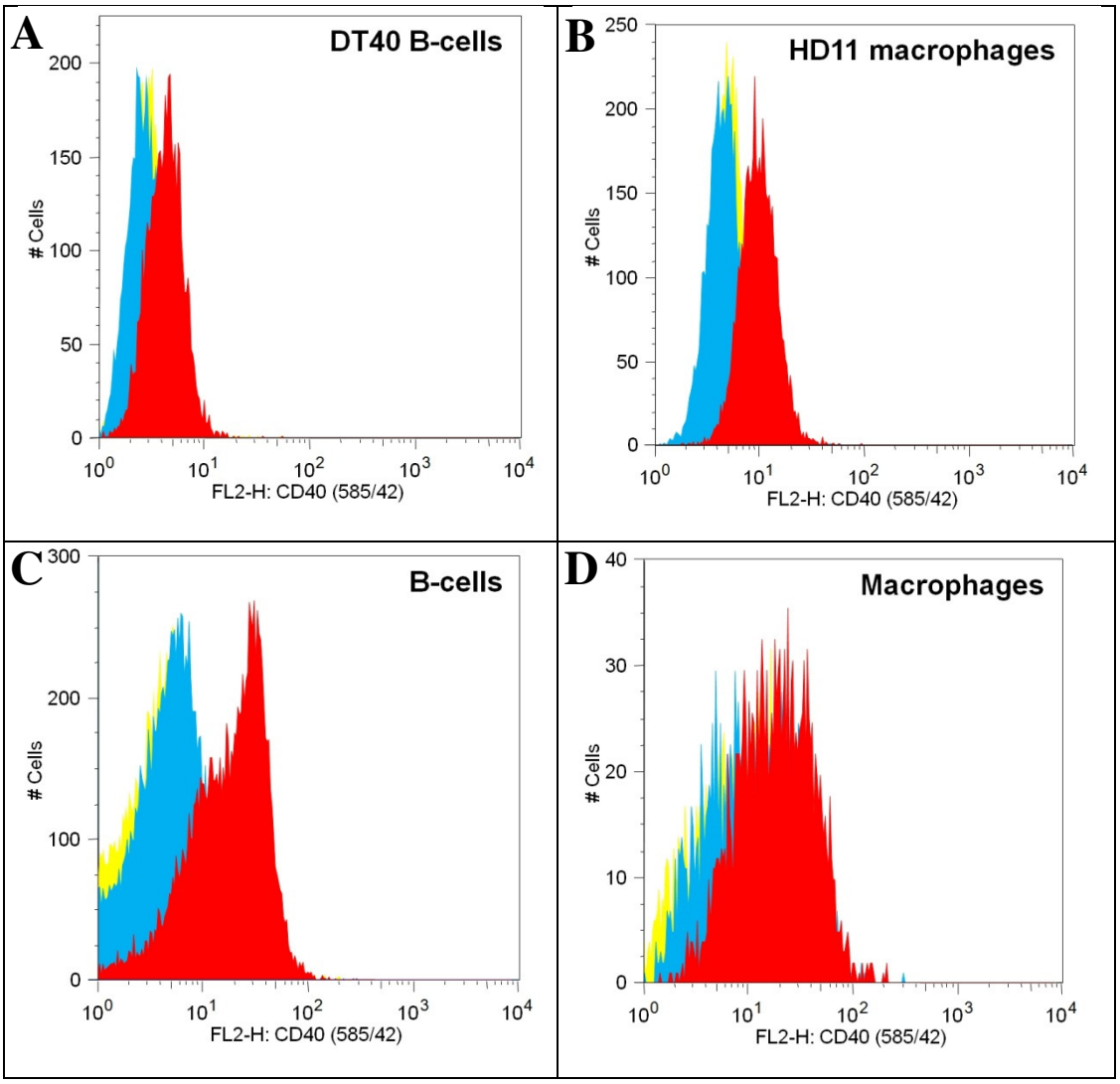

Fig. 5. Flow cytometric assessment of the expression of CD40 on chicken DT40 B-cells, chicken HD11 macrophages, Bu-1 positive chicken primary B-cells, and MHC-II positive chicken primary macrophages (A to D, respectively). Distribution of fluorescence intensities of cells stained with 5µg of either R-Phycoerythrin-labeled 2C5 (red) or R-Phycoerythrin-labeled mouse IgG1 (blue) per one hundred thousand cells. The yellow histogram represents unstained cells.

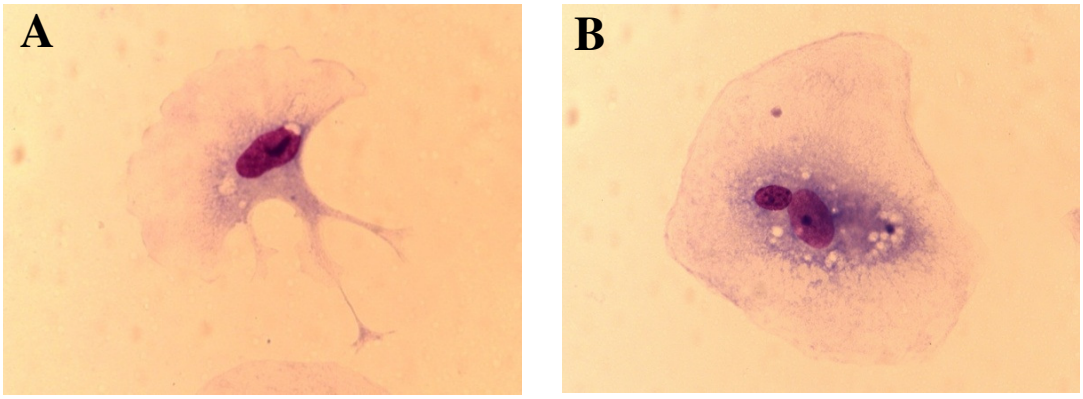

Fig. 6. Morphology of chicken primary macrophages derived from peripheral blood mononuclear cells following Giemsa staining (Figure A and B). Chicken primary macrophages were derived by culture of adherent monocytes isolated from the spleen of a laying hen. This procedure regularly yielded >90% primary macrophages as determined by morphology with Giemsa staining (Nissen-Druey and Speck, 1978; Ramirez et al., 1991). Adherent cells on one 4-chamber slide (Nunc) were fixed by the addition of methanol for 10 minutes, and then stained with 1:50 diluted Giemsa stain solution (Sigma). The diameter of the large cell (right) is 70 µm.

Despite the specific binding of 2C5 to chCD40, chCD40 could not be immunoprecipitated by 2C5 alone. Although the reason for this failure is not entirely clear at present, one possible explanation is that 2C5 may recognize a conformational epitope on chCD40 that is lost upon release of CD40 from the APC cell membrane during the lysis protocol preceding the antigen-antibody interaction. This hypothesis is corroborated by the fact that chCD40 was not detected by 2C5 in Western blotting under reducing SDS-PAGE. By contrast, chCD40 was successfully immunoprecipitated by a pool of five specific anti-chCD40 mAbs, as well as by a mouse polyclonal anti-chCD40 antiserum.

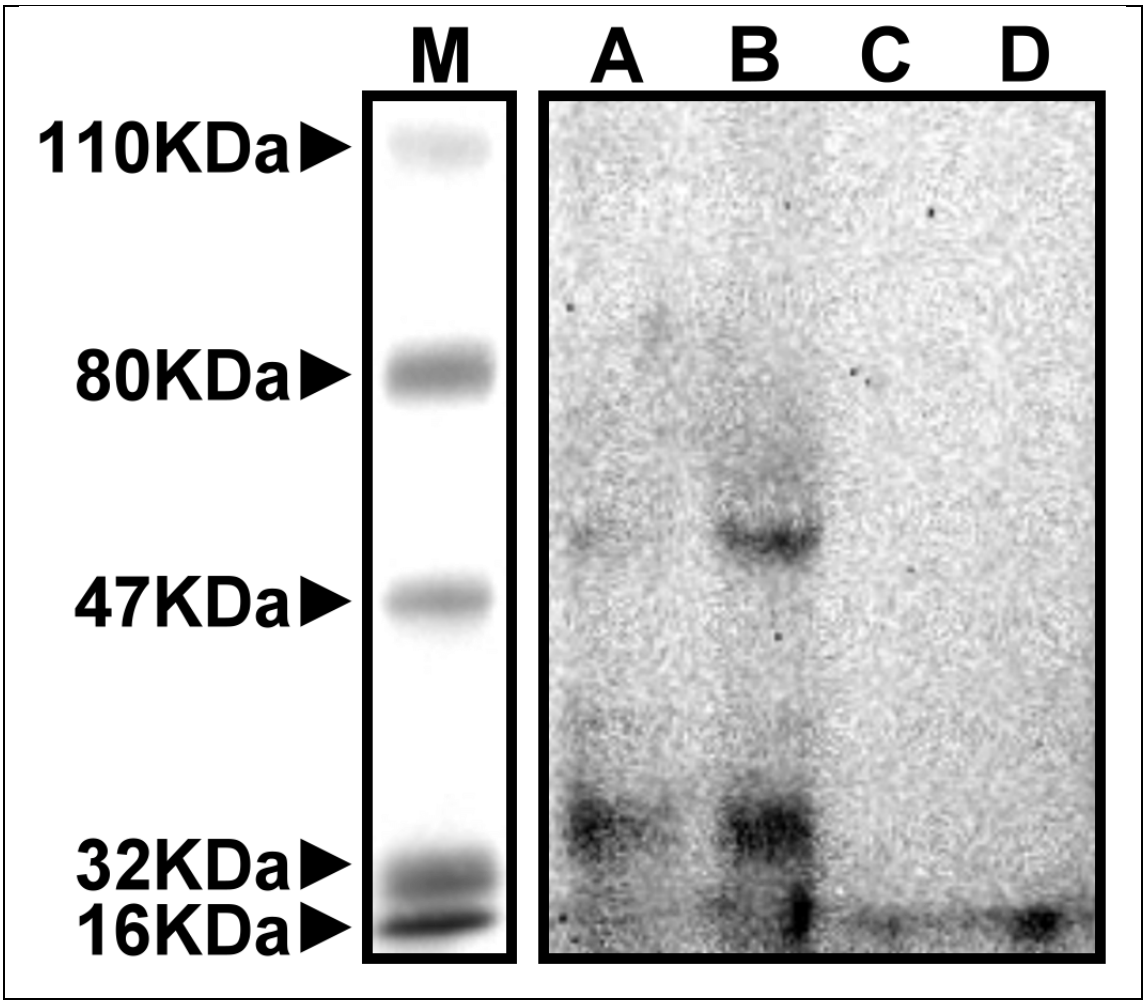

Fig. 7. Immunoprecipitation of chicken CD40 followed by detection with polyclonal anti-chCD40 antiserum by immunoblotting. Chicken CD40 was immunoprecipitated from total cell lysates from chicken DT40 B-cells (lane A) or HD11 macrophages (lane B) using a pool of five agonistic anti-chCD40 mAbs. SDS-PAGE was performed after addition of 25mM of iodoacetamide to the eluted proteins followed by Western blotting. BSA (lane C) and reduced mouse IgG1 (lane D) were used as the negative controls. The membrane was probed with polyclonal antibody raised against the extracellular domain of chicken CD40 followed by (HRP)-conjugated anti-mouse IgG secondary antibody, and visualized with the ECL detection system. In this particular experiment, we used the TrueBlot system (eBioscience) to eliminate the interference by immunoglobulin heavy and light chains originating from the isolated immune complex.

Reducing SDS-PAGE analysis of immunoprecipitated chCD40 from cell lysates showed two bands of 55kDa and 32kDa. Chicken CD40 amino acid backbone accounts for a mass of 30kDa (accession number: Q9DDD2). The extracellular domain of chCD40 consists of 173 residues (43% identity with the extracellular domain of human CD40), including 20 cysteines and three potential N-linked glycosylation sites (Asn25, Asn52, Asn96) (one more

than in human CD40). The discrepancy between the apparent molecular weight demonstrated by SDS-PAGE and the predicted protein mass suggests a carbohydrate content of approximately 41%, likely due to N-linked glycosylation. A similar discrepancy was observed in human CD40 (predicted protein mass = 28.3 kDa *vs.* an apparent molecular weight of 47 kDa estimated by SDS-PAGE) (Paulie et al., 1984; Clark and Ledbetter, 1986; Braesch-Andersen et al., 1989) and was demonstrated to be due to N-linked glycosylation (Braesch-Andersen et al., 1989).

In mammals, agonistic mAbs against CD40 can mimic the effects of CD40L on B-cells *in vitro* (Barr et al., 2003). In this study, agonistic activity of 2C5 was demonstrated by induction of NO synthesis in chicken HD11 macrophages and significant proliferation of serum-starved chicken DT40 B-cells, properties that are shared with chCD40L (Tregaskes et al., 2005; Kothlow et al., 2008). The biological effects of anti-chCD40 mAbs were examined in two different *in vitro* assays: NO synthesis in chicken HD11 macrophages (Tregaskes et al., 2005), and proliferation of serum-starved chicken DT40 B-cells. Anti-chCD40 antibodies from 21 out of 24 anti-chCD40 hybridoma supernatants were capable of stimulating significant NO synthesis in chicken HD11 macrophages. The five best agonistic anti-chCD40 hybridomas were then cloned by limiting dilution. Agonistic anti-chCD40 mAbs secreted from these five clones were purified and used in another round of nitrite detection assays. No detectable NO synthesis was observed by stimulation with mouse IgG1 (1μg/mL), in contrast to all five agonistic mAbs. The mAb designated 2C5 elicited a dose-response NO synthesis in chicken HD11 macrophages with maximal activity at 10μg/mL (Fig. 8A). This mAb was therefore selected for all subsequent assays. CD40 signaling has also been shown to enhance survival and proliferation of chicken DT40 B-cells (Tregaskes et al., 2005). Here we

demonstrated that stimulation of serum-starved chicken DT40 B-cells by 2C5 - in the absence of IL-4 – does result in significant B-cell proliferation (Fig. 8B and 8C) ($P<0.001$), while similar concentrations of control mouse IgG1 had no effect on cell growth. A dose-response relationship was observed with 2C5 doses between 1µg/mL and 10µg/mL. At 1µg/mL, DT40 B-cell densities had increased from the initial seeding density (~$5x10^6$) to ~$6x10^6$ within the first 24 hours.

The activation of chicken HD11 macrophages and chicken DT-40 B-cells at a low concentration (1µg/mL) of 2C5 was shown to occur via the specific binding of this agonistic antibody to CD40 on the cell surface, since control mouse IgG1 had no such effect. In addition to the strong survival promoting activity, the signal delivered by 2C5 was sufficient to induce vigorous proliferation of serum starved chicken DT40 cells in the absence of antigen or cytokines like IL-4. In contrast, mammalian B-cell proliferation stimulated by the cross-linking of CD40 with agonistic anti-CD40 needed the co-stimulation of IL-4 (Clark and Ledbetter, 1986; Clark et al., 1989; Nonoyama et al., 1993).

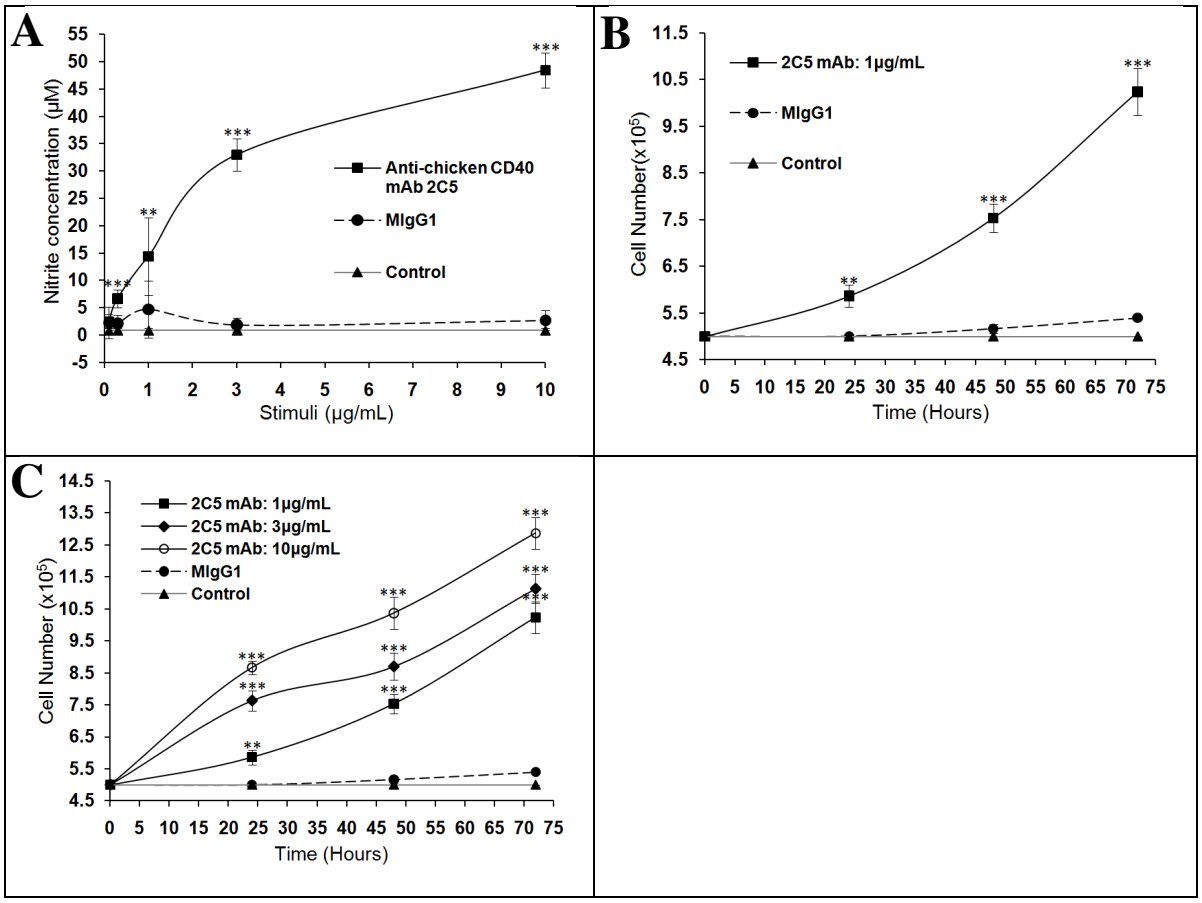

Fig. 8. Agonistic effects of 2C5. A. The vertical axis shows the concentration of NO produced in supernatants from chicken HD11 macrophages stimulated by serial three-fold dilutions of purified anti-chCD40 mAb 2C5 (solid squares), negative isotype control mouse IgG1 (solid circles), or negative control PBS (solid triangles). B. Enhanced proliferation in serum-starved chicken DT40 cells as a function of time. The vertical axis shows the cell density (cells/mL) of live chicken DT40 cells incubated in the presence of 5μg/mL of purified anti-chCD40 mAb 2C5 (solid squares) or negative control mouse IgG1 (solid circles) after 24, 48, and 72 hours. C. Enhanced proliferation of serum-starved chicken DT40 cells as a function of 2C5 concentration. The vertical axis shows the cell density (cells/mL) of live chicken DT40 cells incubated in the presence of serial three-fold dilutions of 2C5 or control mouse IgG1 for 24, 48, and 72 hours. Values are means of triplicate observations ± SD; n=3; *=*P*<0.05; **=*P*<0.01; *=*P*<0.001**

Stimulation of B-cells and activation of APCs such as macrophages by 2C5 is a valuable asset with regard to its potential use in vaccine development for chickens (Barr et al., 2003; Barr et al., 2005; Hatzifoti and Heath, 2007). Barr and co-workers reported that the adjuvant effect of anti-CD40 mAb can be attributed to binding of the antibody to the CD40L-binding domains of CD40 and is mediated by simple direct targeting and stimulation of B-cells, as opposed to interaction with Fc receptors on cells such as macrophages (Barr et al.,

2003). The anti-CD40 mAb-based adjuvant action is extremely potent and may avoid the inflammatory side effects induced by most classical adjuvants (Gendelman et al., 2005; Barr et al., 2006; Hamzah et al., 2008). In mammals, agonistic anti-CD40 mAbs, in combination with Toll-like receptor agonists, have also been reported to activate cell-mediated immunity (Jabara et al., 1990; Schwabe et al., 1997; Khalil and Vonderheide, 2007), since antigen-specific $CD8^+$ T-cells can be elicited by the combination of Toll-like receptor (TLR) agonists and agonistic anti-CD40 mAb (Ahonen et al., 2004; Barr et al., 2005). This approach may provide a useful strategy in the development of novel vaccines against avian as well as mammalian pathogens. The identification of this potent activator of both chicken macrophages and B-cells will be useful in the further study of CD40L/CD40 interactions in the chicken.

Taken together, the binding to CD40 on chicken cells and the stimulation of biological activities leads to the conclusion that 2C5 is functionally equivalent to chCD40L as described by Tregaskes and co-workers (Tregaskes et al., 2005; Kothlow et al., 2008). The identification of chCD40 by 2C5 further allowed investigation of the chicken CD40L/CD40 system and comparison with its mammalian counterpart.

CHAPTER IV

PROOF OF CONCEPT: *IN VIVO* TARGETING A PEPTIDE TO CHICKEN CD40

Introduction

For over a hundred years, we have known that immunized hens transfer IgG from serum to egg yolk, but this alternative possibility of producing antigen-specific IgG in yolk has only attracted attention from last decades (Gassmann et al., 1990). The main advantage of producing antibodies in chicken eggs is the convenient acquisition of abundant amounts of antigen-specific IgG (>1g per week) without venipuncture (Svendsen Bollen et al., 1996). Yolk is an excellent and easily accessible source of IgG, and fewer resources are required for collecting eggs than for bleeding animals (Tan et al., 2012). As a consequence of phylogenetic separation of chickens from the reptile ancestor of mammals about 300 million years ago, many studies have indicated that chicken antibodies recognize more epitopes, especially highly conserved proteins, than mammalian antibodies (Camenisch et al., 1999). In many solid phase immunoassays, using chicken antibodies can effectively increase detection accuracy because they neither interact with mammalian rheumatoid factors nor activate mammalian complement systems, which can greatly reduce the possibility of false positive results or interference from complement components (Larsson et al., 1993). Furthermore, chicken antibodies do not react with protein A/G and exhibit low cross-reactivity with mammalian antibodies because of their structural differences. This eliminates unwanted reactions in immunological assays, especially sandwich type immunoassays utilizing anti-species antibodies (Larsson et al., 1992).

Although short peptides congenitally suffer from a serious general drawback of low immunogenicity, chickens immunized with peptide-carrier conjugate still produce antibodies that recognize the corresponding native epitopes (Nagase et al., 1979). Immunization of chickens with a peptide-carrier conjugate is usually carried out by repeated intramuscular injection in each of the breast muscles with the antigen emulsified in complete Freund's adjuvant or precipitated in alum (Gassmann et al., 1990; Ohta et al., 2009). However, antigen-specific IgG response can only be observed three weeks after the first priming, and maintenance of high IgG titer still requires repeated boosts. To elicit a quick and strong specific IgG response in chickens against peptide antigens, the use of more potent immunological adjuvants is considered one of the most promising ways among current immunization strategies (Capua et al., 2003). Many methods for improving and speeding antibody responses against injected antigen have been evaluated in mammals. One method is to target the antigen directly to antigen presenting cells (APCs) through the use of antibody or chemokine *in vivo* (Berry et al., 2003; Hatzifoti and Heath, 2007; Behzad et al., 2012). Agonistic antibodies against CD40 have great potential as immunological adjuvants. Without the use of carrier protein, recent research has shown that administration of agonistic anti-CD40 monoclonal antibodies conjugated with peptide/hapten can induce strong and specific antibody responses in mammals (Hatzifoti and Heath, 2007). In previous chapter, the cross-linking of CD40 expressed on the surface of chicken APCs with agonistic anti-chicken CD40 mAb 2C5 was able to activate their functional status (Chen et al., 2010). In this chapter, chCD40 targeting of a synthetic short peptide as a method for rapid production of peptide-specific and isotype-switched chicken IgG was evaluated. This study was designed as a proof-of-concept battery cage study with Leghorn chickens reared until seven weeks of age.

The generation of rapid and isotype-switched antibody responses by *in vivo* APC targeting in chickens should facilitate producing antigen-specific IgG in eggs.

Materials and methods

Streptavidin mediated complexing of peptide to mouse antibody

The antibody-peptide complex used for chicken immunization was prepared by utilizing the specific interaction of biotin with streptavidin as illustrated in Fig. 9. A synthetic peptide, NAWSKEYARGFAKTGK, with addition of biotin on the amino-terminus of N residue was commercially obtained (GenScript Inc., Piscataway, NJ). Streptavidin (SA) (Thermo Scientific) was used for a controlled complexing of biotinylated peptide to biotinylated mouse antibody (MIg). 2C5 was purified from ascites fluid by protein G immunoaffinity chromatography. To prepare biotinylated MIgs, the *cis*-glycol groups of carbohydrates on the Fc region of MIg were oxidized by periodate to form reactive aldehyde groups, which were then actively linked to the hydrazide activated biotin. Briefly, 2C5 or control MIg was dialyzed against oxidation buffer (0.1M sodium acetate, pH 5.5) at 4^{o}C overnight, and one part (v/v) MIg in oxidation buffer was then incubated with one part (v/v) oxidation buffer containing 20mM sodium *meta*-periodate (Thermo Scientific) at room temperature for 30 minutes. The reacted MIg was then dialyzed against coupling buffer (0.1M sodium phosphate, 0.15M NaCl, pH 7.2) to remove excess periodate. The direct biotinylation on the Fc region of MIg was then achieved by incubating nine parts (v/v) of the oxidized and buffer-exchanged MIg solution with one part (v/v) of freshly prepared 50mM EZ-Link® Biotin-LC-Hydrazide (Thermo Scientific) in dimethyl sulfoxide. This reaction was allowed to proceed for two hours at room temperature. Biotinylated MIg was separated

from non-reacted material by dialysis against phosphate buffered saline (PBS), pH 7.4. To produce SA-MIg complex, SA was incubated with biotinylated MIg at molecular ratio of 1:2 in PBS while stirring at 4°C for three hours. The SA-MIg complex was then further incubated with biotin-peptide at molecular ratio of 1:2 in PBS while stirring at 4°C for three hours. The MIg-SA-peptide complex was sterile filtered and stored at 4°C until used. The incorporation of all necessary components in the final complex and stoichiometric ratio of the complex formation were evaluated by matrix-assisted laser desorption/ionization in the Laboratory for Biological Mass Spectrometry at Texas A&M University. The antigen-binding capacity of the 2C5-peptide complex was tested for binding to the rchCD40$_{ED}$ by ELISA

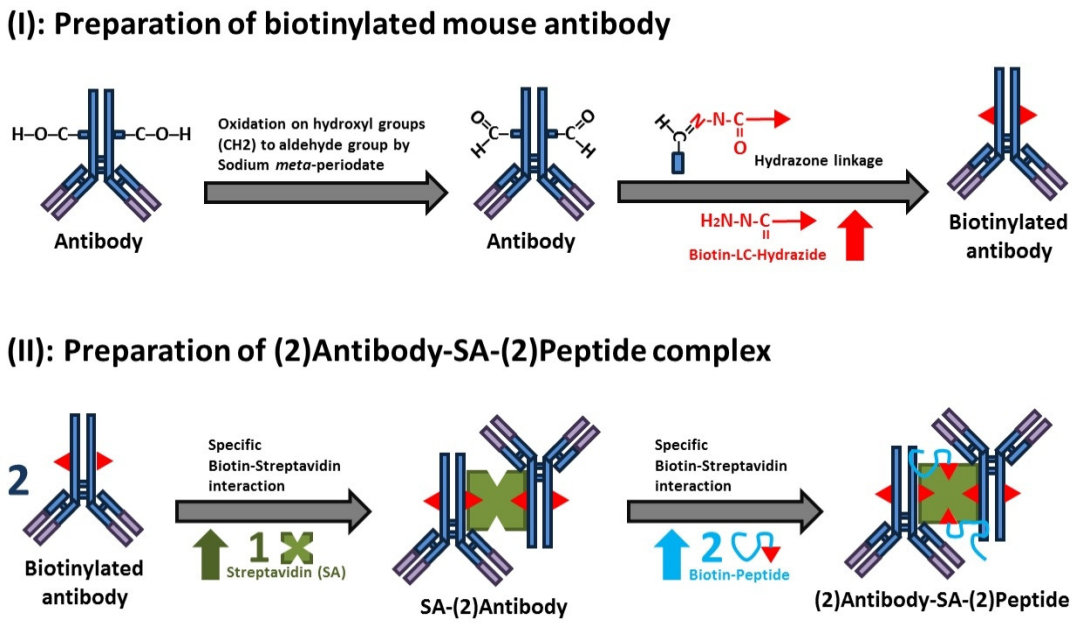

Fig. 9. Preparation of antibody-peptide complex utilizing specific biotin-streptavidin interaction. (I): Biotinylation was limited to the carbohydrate groups on the Fc region of MIg, hence without interfering with antigen-antibody interaction. (II): Streptavidin (SA) was used for controlled complexing of biotinylated peptide with biotinylated MIg. Mab 2C5 in the 2C5-SA-peptide complex retains biological function demonstrated by ELISA.

Administration of mAb 2C5-peptide complex on chickens

The conditions for animal use here were approved by the Institutional Animal Care and Use Committee of Texas A&M University, in accordance with the guidelines of the American Association for Laboratory Animal Science. Five-week old Leghorn chickens were wing-banded and then randomly allocated to different groups (n=7). Chickens were immunized subcutaneously (s.c.) in the nape of the neck with 2C5-peptide complex or MIgG-peptide complex control at three dose levels (10µg, 30µg, and 90µg) in a volume of 0.5ml emulsified PBS [5% (v/v) squalene, 0.4% (v/v) Tween 80 (SIGMA) in PBS)] per chicken. In addition, groups of chickens were immunized s.c. with SA-peptide complex (omission of the antibody component). Finally, one experiment involved comparison of adjuvant effect of 2C5 and alum. Finally, three groups of chickens (n=7) were immunized s.c. with three dose levels (1.5µg, 4.5µg, and 13.5µg) of SA-peptide complex precipitated in Imject Alum Adjuvant (Thermo Scientific). Serum samples were collected from the wing vein on days 4, 7, 10, and 14 post-immunization (p.i.) for determination of peptide-specific IgG responses by ELISA.

Quantification of peptide-specific serum IgG in by ELISA

Serum levels of peptide-specific IgG were determined by ELISA. First, biotin-peptide was incubated with goat anti-biotin antibody (Thermo Scientific) on a rotator at 37^{o}C for one hour. Flat-bottom, 96-well microtiter plates (Thermo Scientific) were coated with peptide-goat antibody complex (5µg/mL) in 0.05M carbonate-bicarbonate buffer, pH 9.6, overnight at 4^{o}C. Supernatants containing unadsorbed peptide-goat antibody complex were removed, and plates were blocked with PBS containing 5% (w/v) bovine serum albumin (BSA)

(Rockland Immunochemicals Inc., Gilbertsville, PA) for one hour at 37°C. Peptide-goat antibody coated wells were washed with PBS containing 0.2% (v/v) Tween 20 (SIGMA) (PBST) and then incubated with chicken serum samples diluted (1:100) in PBST containing 3% (w/v) BSA overnight at 4°C. The plates were then washed as described above and incubated with horseradish peroxidase conjugated rabbit anti-chicken IgY (H+L) (Thermo Scientific) diluted (1:12,000) in PBST containing 3% (w/v) BSA for one hour at room temperature. The color reaction was developed by using OptEIA™ TMB substrate (BD) per the manufacturer's instructions, and terminated by addition of 1N sulfuric acid. Absorbances at 450 nm [A(450)] were measured in a Wallac plate reader (PerkinElmer Inc., Waltham, MA). The presence of peptide-specific IgG was determined by relating the mean A(450) value of each serum sample to that of the chicken peptide positive control serum (1:100). The relative levels of peptide-specific IgG in all serum samples were determined and normalized by calculating the sample to positive (S/P) ratio as follows: S/P value = (Sample mean - negative control mean)/(Peptide positive control mean - negative control mean). Comparison of means of S/P values was performed using one-way analysis of variance (ANOVA) with least significant difference (L.S.D.) as multiple comparison tests. Student's t-test was used to determine significant differences in means of S/P values between treatments across all groups, and S/P values of the SA-peptide complex group were used as baseline. All data were analyzed and generated using JMP® version 9 software (SAS Institute Inc., Cary, NC). Statistical significance was determined at $P < 0.05$.

Results and discussion

Mammals immunized with conjugates of agonistic anti-CD40 mAb and peptides produce enhanced antigen-specific antibody responses, manifested by high titer, affinity matured, and isotype-switched antibody in serum (Berry et al., 2003; Hatzifoti and Heath, 2007). Utilizing these strategies for facilitating the rapid production of such highly desirable antibody responses in chickens has not been previously investigated. In chapter I, we have shown that administration of 2C5 mimicked the effects of CD4 T-cell help, enabling direct activation on chicken APCs *in vitro*, even in the absence of cytokine (Chen et al., 2010).

The recombinant streptavidin was used as the carrier moiety and complexed to biotinylated 2C5 / biotinylated peptide. A controlled complexing was mediated using specific interaction of biotin and streptavidin. Complexing efficiency was evaluated by a shift in molecular weight of the streptavidin on SDS-PAGE gels and confirmed using mass spectroscopy. The average molecular weight of streptavidin with empty complexing groups was about 53 kDa, and the size shifted to 57 kDa at average as predicted while two biotinylated peptides (2.1 kDa) were bound to streptavidin (Fig. 10). We estimate that the average valence of biotinylated peptide bound to one streptavidin is approximately two and this 2(peptide)-streptavidin complex still have two binding sites for biotinylated mouse immunoglobulins.

In order to assess functional integrity of CD40 binding, binding to chCD40 of mAb 2C5-peptide complex was assessed by ELISA. Complex, bound to chCD40 was detected using anti-SA antibody (SIGMA). ELISA readout indicated that specific chCD40 binding ability was intact in mAb 2C5-peptide complex and absent in MIg control complex and SA-peptide complex.

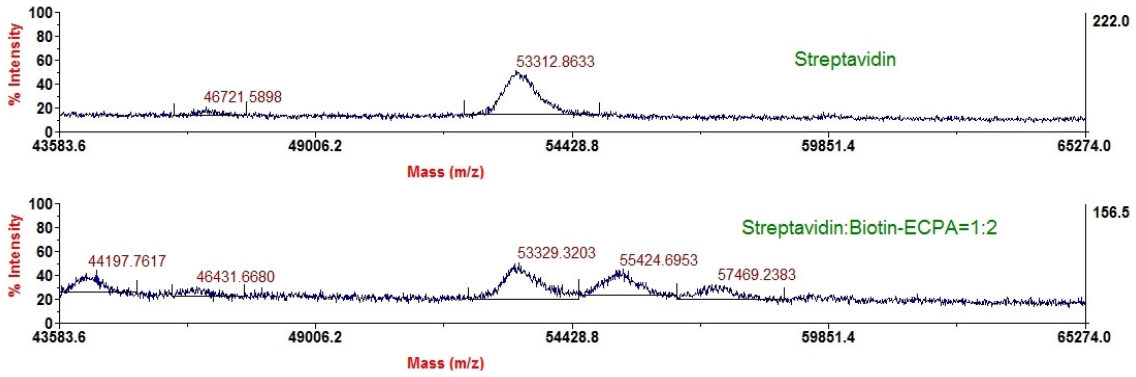

Fig. 10. Overlay of the streptavidin-biotinylated peptide complex analyzed in mass spectroscopy. The upper line represents the streptavidin in PBS but without biotinylated peptide attachment. The lower line represents the complex that is composed of one streptavidin and two biotinylated peptide.

To evaluate the adjuvant effect of 2C5, groups of five-week old chickens were immunized once s.c. with 10µg, 30µg, or 90µg mAb 2C5-peptide complex, and compared with the various control groups. Additional groups of chickens received same doses of SA-peptide complex precipitated in alum or dissolved in emulsified PBS. Serum samples were collected from all immunized chickens at day 4, 7, 10, and 14 p.i.., and peptide-specific IgG response was assessed by ELISA. As shown in Fig. 11, 2C5 significantly (P<0.001) enhanced peptide-specific IgG responses compared to controls 4 days p.i... While the general profile of the peptide-specific IgG response was the same with the three doses tested, the 30µg dose of mAb 2C5-peptide complex produced the optimal response in terms of magnitude and duration. Interestingly, the response was not improved by increasing the dose to 90µg.

A lower quantity (10µg) of mAb 2C5-peptide complex was sufficient to elicit significant (P < 0.001) peptide-specific antibody response. By contrast, no peptide-specific IgG response was found in chickens immunized with either SA-peptide complex in emulsified PBS or precipitated in alum (Fig. 11). This is consistent with other studies, which reveal the superiority of chicken CD154 compared to alum adjuvants (Pose et al., 2011).

Moreover, low immune response has been reported in chickens using alum as adjuvant at early stages (Murthy et al., 2007). The undetectable immune response when using alum could be due to the fact that only peptide-specific IgG response, not total antibody titers were examined.

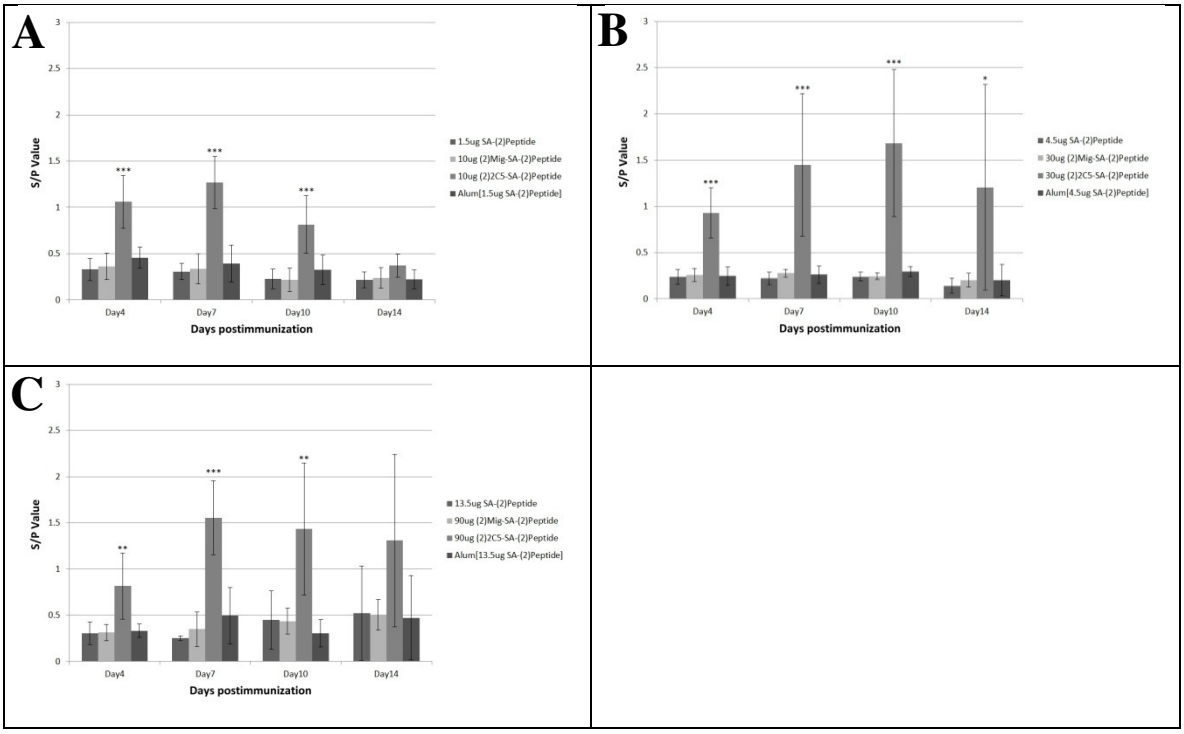

Fig. 11. Peptide specific IgG response elicited by 2C5 as adjuvant determined by ELISA. Groups of seven 5-week-old male Leghorn chickens were subcutaneously vaccinated once with 10μg (A), 30μg (B), or 90μg (C) mAb 2C5-peptide complex or controls. To compare the adjuvant effect of 2C5 and alum, serum samples were collected from chickens immunized (once, s.c.) with the same amount of SA-peptide complex in emulsified PBS or precipitated in alum, and peptide-specific IgG response was assessed by ELISA. In each case, error bars represent standard deviations from the mean and the asterisks represent statistical significance (n=7; *P<0.05; **P<0.01; ***P<0.001) compared with SA-peptide complex controls as determined by Student's t-test.

Generally, 10–100μg protein antigen emulsified/precipitated in adjuvant is usually applied intramuscularly in the breast muscle at two or three injection sites of 7 to 8-week-old chickens, and at least 2μg antigen are needed to induce significant antibody responses in five week old chickens (Cornelissen et al., 2010). However, a very low amount of peptide (0.2μg)

was sufficient to elicit significant ($P<0.001$) peptide-specific IgG response in this study. This is in clear contrast the slow, short-lived IgM response obtained with classical adjuvants (Cornelissen et al., 2010; Liu et al., 2011). The adjuvant effect of 2C5 was found to be substantially more potent than the commonly used alum.

Since accelerated, enhanced, isotype-switched IgG response against peptide was only found in chickens immunized with mAb 2C5-peptide complex, the activation on APCs, especially peptide-specific B-cells, is attributed to CD40 targeting, not to danger-associated molecular patterns through Fc receptor positive cells. Besides the direct activation on B-cells, the adjuvant effect of 2C5 may also include promotion of antigen presentation of APCs and boosting of CD4+ T-cell help. We conclude that this enhanced immunogenicity is due to the engagement of 2C5 with CD40 on APCs *in vivo*, by which directly activates peptide specific B-cells. In the meantime, this engagement of CD40 with the 2C5 portion in the mAb 2C5-peptide complex may also be facilitated on peptide-specific B-cells while peptide-specific BCR on their surface held the complex at the same time. Our results are in accordance with previous observations where chickens were immunized with CD154-antigen chimeric protein, and seen to be the result of an improved antigen presentation and boosting of CD4+ T-cell help, which plays a direct role in modulating B-cell response (Sanchez Ramos et al., 2011). This effect may be related to a better employ of this peptide by enhancing the engagement of 2C5 on the complex for B-cell activation simultaneously with CD40 on the surface of peptide-activated B-cells to induce the peptide-specific IgG response (Li, 2005). Our results also suggest that the mAb 2C5-peptide complex may react with APCs immediately at the injection site after inoculation, because no visible lesion was found on the injection site within two weeks PI. For the concern of animal welfare, 2C5 can be considered as a safe

adjuvant for chicken and Freund's adjuvant is no more needed, Freund's adjuvant is still the gold standard though.

Here we need to argue the versatility of this approach. By directional biotinylation on carbohydrate group on Fc region of antibodies, the binding of SA to biotin can only occur on Fc region of antibodies. This interaction does not affect the specific binding of chCD40 to 2C5 on mAb 2C5-peptide complex, and interactions of Fc fragment of 2C5 with Fc receptor positive cells may also be avoided. Biotinylation is easy (a myriad of reagents commercially available), fast and causes minimal chemical changes in the hapten. Every possible biomolecule can be easily biotinylated, thus simple mixing with the mAb 2C5-SA complex makes the immunogen ready for injection. Since chicken antibodies tend to recognize more epitopes than mammalian antibodies, our *in vivo* APCs targeting strategy can clearly be useful for most T-dependent antigens. Because of the highly efficient and easy complexing technology reported here, T-independent antigens like carbohydrates, steroids or nucleotides should also be applied in this system just by the addition of biotin for a controlled complexing. This is especially important when production of antibodies against highly conserved mammalian proteins, such as prions, is needed. Since chicken mucosal tissues are rich in APCs, we also expect this 2C5 based biological complex to induce efficient systemic and mucosal immune responses through the mucosal immunization route. Here we conclude that *in vivo* CD40 targeting antigen on APCs can enhance the immunogenicity of synthetic peptides, and can be an effective method for the rapid production of IgG in yolk.

This study has facilitated the design of approaches to apply anti-chCD40 mAb while it attempts to improve the effects of subunit vaccines against a variety of chicken diseases. However, CD40 is not the only pathway to activate B-cells in chickens (Hobbs et al., 2006;

Stupar and Peterson, 2006; Totlani and Peterson, 2006). Other soluble costimulatory molecules, like another TNF ligand superfamily member (chBAFF) (Schneider et al., 2004), cytokines (chIL-4) (Peterson and Urquhart, 2006), chemokines (chIL-8) (Krumholz et al., 2006), dendritic cell marker (CCR7) (Wu et al., 2011) are also reported to be necessary for B-cell activation, isotype switching, or formation of memory B-cells, thereby are good candidates for the enhancement of synergistic effect with CD40 to activate B-cells. Agonistic anti-CD40 mAbs that were in combination with Toll-like receptor agonists have also been reported to activate antigen specific cell-mediated immunity (Peterson et al., 2006b; Shi et al., 2006). Hence, to maximize the synergistic effects on B- and T-cell immunity for chickens against pathogens, combination of these soluble mediators with agonistic anti-chCD40 mAbs offers practical advantages in current immunization programs for the poultry industry.

The poultry industry is in urgent need of one-shot vaccines because it helps to save budget on labor fees and labor costs, time, and other overheads in traditional vaccination programs (Goforth et al., 2006; Mitchell et al., 2006; Peterson and Krauss, 2006). Additionally, time for the production of protective antibodies in chickens can be shorten for several weeks by one-shot vaccine because of the enhanced adjuvant effects, like significant enhancement of antigen specific antibody response was induced by 2C5 within four days PI, providing quick protections against specific pathogens. However, despite the benefits of safety and efficiency, mass production of 2C5 based complex for vaccination in the poultry industry is least cost-effective. Adenoviral vectors expressing chimeric variants of antigen fused to the 2C5 may overcome cost and production issues.

Taken together, similar to what occurs in mammalian systems, our results concluded that CD40-targeted antigen delivery is an effective strategy to improve the kinetics of adaptive

immunity on subunit vaccines in chickens. Immunopotentiation mediated by exogenous 2C5 in chickens is antigen dependent, as noted for other adjuvants and other species. Not only the observed positive effect on induction with this 2C5 based complex might be used to resolve an established NE, but also to be utilized to develop more effective strategy for subunit vaccine development against avian pathogens, especially for avian influenza and infectious Bursal disease.

CHAPTER V

IN OVO ADMINISTRATION OF ADENOVIRUS-VECTORED CD40 TARGETING VACCINE AGAINST *C. PERFRINGENS* α-TOXIN

Introduction

Modern broiler chickens all over the world have been threatened since the appearance of necrotic enteritis (NE) in 1961 (Parish, 1961), which is generally believed to be mainly caused by α-toxin secreted by *Clostridium perfringens* type A (Cooper and Songer, 2009). It is one of the most economically important enteric diseases in broiler chickens, which results in outbreaks with substantial mortality. *C. perfringens* is a spore forming Gram-positive anaerobic bacteria that can be found in the soil, feed, and intestine of chickens, but they are more commonly isolated in large numbers in poultry farms (Lee et al., 2011). *C. perfringens* is a normal inhabitant of chicken intestine in low numbers, which has no apparent negative impact on chickens. However, *C. perfringens* becomes pathogenic under some predisposing circumstances, such as coccidiosis, mycotoxins, and environmental stress (McReynolds et al., 2004). Under these circumstances, *C. perfringens* type A can cause a spectrum of diseases in chickens including NE, cholangiohepatitis, diarrheal illness, and some subclinical / mild diseases, which results in depression of the flock growth rate and high economic losses (Williams, 2005). The standard approach in the poultry industry to protect chickens against these diseases is through the use of antibiotic feed additives, which have been used for many years but are now forbidden (Lee et al., 2011). As a consequence, NE has now become a serious threat to poultry producers, and antibiotic-free poultry products are highly demanded on markets; it is therefore essential to investigate methods to efficiently minimize exposure

of chickens to pathogenic factors of *C. perfringens*, and improve clinical efficacy of currently available vaccination strategies against NE. Immunization of broiler chickens with *C. perfringens* toxoid vaccine helps reducing the incidence and severity of NE and protecting intestinal integrity against clostridial infection (Saleh et al., 2011). Vaccination seems to be an interesting and successful alternative for the control of NE in broilers without the use of antibiotic feed additives. To vaccinate chickens in great numbers within a short time frame in response to an outbreak of NE, practical vaccines have to be produced rapidly and inexpensively, and have to elicit protective immunity in a single-dose regimen without involving labor-intensive procedures. Using vectored adenovirus vaccines for *in ovo* vaccination covers all these requirements (Tatsis and Ertl, 2004).

Vectored adenoviruses have tremendous potential in both research and therapeutic applications, especially for expression of recombinant proteins in host cells (Hermening et al., 2004). Introducing recombinant DNA coding for interesting antigens into host cells by adenovirus provides many advantages. First, adenovirus remains epichromosomal after entering cells, which does not activate or inactivate host genes (Zhang et al., 1994). Second, replication-defective adenovirus, such as vectored adenovirus type 5 (Ad5), can be produced at a large scale within a short period at low cost (O'Brien et al., 2009). Finally, Ad5 can transduce either dividing or non-dividing cells through many routes (Avakian et al., 2007), which is very useful for *in ovo* vaccination strategies. Another advantage of using Ad5 as vaccine is that the hexon protein on adenovirus is highly immunogenic and can efficiently elicit innate immune responses. Production of pro-inflammatory cytokines can be enhanced in the immunized animal, which then promotes activation, development, and differentiation of dendritic cells. Mature dendritic cells can further activate antigen-specific CD4 and CD8

T-cells, and then adaptive immunity can be greatly enhanced (Zandvliet et al., 2010). High administration doses (1×10^8 to 5×10^8 infectious units) for a single vaccination is practical when using Ad5, which allows subunit vaccines to overcome their inherently poor immunogenicity (Josefsberg and Buckland, 2012).

Ad5 has also emerged as a promising alternative to conventional vaccines for *in ovo* administration because the use of recombinant adenovirus could eliminate the need for booster vaccinations and post-vaccination reactions or local reactions typical caused by vaccines made from live or inactivated organisms (Williams and Hopkins, 2011).

In ovo vaccination with Ad5 expressing antigens of high pathogenic avian influenza virus (HPAIV) has been assessed, and protective immune responses against HPAIV were reported in several studies (Singh et al., 2010; Breedlove et al., 2011; Mesonero et al., 2011). These *in ovo* conventional Ad5 vaccines against HPAIV have been considered to be more effective, cheaper, and safer than classic subunit vaccines (Josefsberg and Buckland, 2012). *In ovo* vaccination with Ad5 also eliminate the possibility of interference with the maternal immunity and/or other possibilities of attenuated viral vaccines from becoming latent in vaccinated birds. Using Ad5 vaccines also allows the use of the Differentiating Infected from Vaccinated Animals (DIVA) strategy because no possibility of recombination reaction will occur between recombinant Ad5 and target pathogens, especially pathogenic bacteria, in the environment (Capua et al., 2004).

One of the methods to increase immunogenicity of Ad5 vaccine is using molecular adjuvants (Mesonero et al., 2011). The use of molecular adjuvants, like chicken CD154, is also suggested to give Ad5 vaccine the ability to induce quick protective humoral responses under emergency conditions, even though *in ovo* vaccination (Breedlove et al., 2011). CD154

is a glycoprotein expressed as a type II integral membrane protein mainly on activated T-cells (van Kooten and Banchereau, 2000). This molecule belongs to the TNF superfamily and has been defined as the most important co-stimulator for activating APCs, especially for B-cells, through the CD40 signaling pathway (Zan et al., 1998). The interaction of CD40-CD154 is also necessary for clonal expansion, germinal center formation, isotype switching, and formation of memory on B-cells (Banchereau et al., 1994). Several studies have shown that murine CD154 administered individually with T-cell dependent /independent antigens, or as a fusion protein can function as a molecular adjuvant, which enhances adaptive immunity and protection against diseases (Mackey et al., 1997). In avian species, the adjuvant effect of CD154 was first demonstrated by immunization of ducks with hepatitis B virus mixed with duck CD154, which was used as a model for infection of humans with hepatitis B virus (Gares et al., 2006). Several studies have shown that chicken CD154 (chCD154) can act as a molecular adjuvant enhancing the immune response (Layton et al., 2009; O'Meara et al., 2010). Additionally, significant specific humoral and cellular immune responses against HPAIV were induced in chickens immunized with the Ad5 expressing chCD154-hemagglutinin (HA) chimeric protein (Naskalska et al., 2009; Mesonero et al., 2011). Cellular immune responses and HA-specific antibody titers were significantly higher than those induced by Ad5 expressing HA alone (Mesonero et al., 2011).

Agonistic mAb/scFv against chCD40 have great potential as immunological adjuvants, and its partial functional equivalence with chCD154 has been demonstrated (Chen et al., 2010). We have shown that agonistic monoclonal anti-chCD40 antibody (designated 2C5) could induce significant isotype switched antibody responses against a hapten that was biologically complexed to 2C5 only 4-days post-immunization (Chen et al., 2012). Based on

strong scientific evidence and extensive experience with clostridial vaccines for mammals, some recent studies have been carried out on *C. perfringens* type A/C toxoid and have shown promising results (Saleh et al., 2011). However, mice immunized intraperitoneally with a recombinant C-terminal of Cpα ($Cp\alpha_{CD}$) had transient and localized lesions compared to sham immunized mice upon challenge with *C. perfringens* type A (Titball et al., 1993). After a high dose challenge (3.74×10^9 cfu), there were no survivors in the control group, whereas 90% of the mice in the immunized group survived. These results not only demonstrate the usefulness of the $Cp\alpha_{CD}$ as an immunogen for inducing protective immune responses, but also suggest its candidacy for vaccine development other than toxoids (Stevens et al., 2004).

In this chapter, the adjuvant capacity of agonistic anti-chCD40 scFv was combined with a delivery system based on an adenoviral vector to create a vaccine [Ad-(CD6-DAG1-$Cp\alpha_{CD}$-FLAG)] for *in ovo* vaccination, which can potentially induce immunoneutralizing antibodies against Cpα. With the same adenoviral vector platform previously evaluated in many studies on AIV by *in ovo* administration, DAG1-$Cp\alpha_{CD}$-FLAG can be expressed and secreted by transduced chicken embryonic cells, and then actively target CD40 on APCs. We postulated that the agonistic anti-chCD40 scFv fused to $Cp\alpha_{CD}$ gene could bind to CD40 on the cell surface of chicken APCs, especially B-cells, and enhance their activation and differentiation through the well-characterized CD40 signaling, in order to induce quick and strong $Cp\alpha_{CD}$-specific IgA responses. The efficacy of *in ovo* vaccination of broilers with Ad-(CD6-DAG1-$Cp\alpha_{CD}$-FLAG) in controlling the NE was evaluated by *C. perfringens* type A challenge 18 days post-hatch.

Materials and methods

Development of agonistic scFv against chCD40

The agonistic anti-chCD40 scFv diabody (designated DAG1) used in this study was kindly provided by Dr. Daad Abi-Ghanem (Department of Poultry Science, Texas A&M University). Briefly, reverse transcription was used to clone cDNA from the splenocytes harvested from mice hyper-immunized against recombinant $chCD40_{ED}$ and the genes encoding the V_H and V_L domains were amplified by separate asymmetric PCR. V_H and V_L domains then linked by overlap-extension PCR and this anti-chCD40 scFv gene was modified to contain sequences encoding CD6 secretory signal sequence and HA-tag at its 5' and 3'-end, respectively. The resultant anti-chCD40 scFv was designated as DAG1. Binding of DAG1 to chCD40 was verified by immunocytochemical staining on cells constantly expressing full-length chCD40 using purified DAG1 and an alkaline phosphatase conjugated anti-HA secondary antibody (Roche, South San Francisco, CA) as detecting reagents. DAG1 was further cloned into a pCMV vector for expression in FreeStyle™ 293 cells, and the biological function of purified DAG1 was assessed through NO production in HD11 macrophages and agglutination of DT40 cells (Abi-Ghanem et al., 2011).

Cloning of CD6-DAG1-$(G_4S)_3$

Primers used to amplify the gene encoding CD6-DAG1 with the addition of hydrophobic linker $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_3$ $[(G_4S)_3]$ at its 3' end are listed in Table 2. The reverse primer DAG1-$(G4S)_3$-Rev introduced the underlined nucleotides encoding a hydrophobic linker $(G_4S)_3$, which was used for linking DAG1 to $Cp\alpha_{CD}$-FLAG. The CD6-DAG1-$(G_4S)_3$ was amplified by PCR using pCMV-(CD6-DAG1-HA) as template and primer

pairs of SmaI-CD6-Fwd / DAG1-$(G4S)_3$-Rev. Briefly, 100ng pCMV-(CD6-DAG1-HA) and 2.5µL of each primer (10µM) were added into 25µL Phusion High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA) in a PCR tube, and the final volume was adjusted to 50µL with MQ-H_2O. The amplification reaction was run in a thermal cycler with the following program: initial denaturation at 98°C for 30 seconds, 40 cycles consisting of denaturation at 98°C for 15 seconds, annealing at 55°C for 15 seconds, and extension at 72°C for 45 seconds, and was ended with a final extension step of 10 min at 72°C. PCR product with the expected size (933bp) was agarose gel purified and used for linking to $(G_4S)_3$-$Cp\alpha_{CD}$-FLAG.

Table 2: Primers for cloning CD6-DAG1-$(G_4S)_3$	
Primers	Sequence (5'→3')
SmaI-CD6-Fwd	CCCGGGACCATGCCCATGGGGTCTCTGCAA
DAG1-$(G_4S)_3$-Rev	CCGCCAGAGCCACCTCCGCCTGAACCGCCTCCACCA GAAGCGTAGTCCGGAACGTCGTAC

Generation of irrelevant control scFv diabody: CD6-3G4 diabody

3G4 scFv monomer (designated as $3G4_m$) was prepared using cDNA generated from a hybridoma that secretes a Neospora caninum apical antigen-specific mouse IgG_{2b} mAb 3G4/20 (Baszler, T. V. 2004. unpublished). In this study, 3G4 monomer was modified to a diabody format as control scFv (designated as $3G4_d$) similar to the DAG1 diabody. pCMV-(CD6-$3G4_m$-FLAG) was kindly provided by Dr. Waithaka Mwangi (Department of Veterinary Pathobiology, Texas A&M University) as template for cloning of CD6-$3G4_d$. Primers used to modify CD6-$3G4_m$-FLAG to CD6-$3G4_d$-$(G4S)_3$ are listed in Table 3. For cloning the V_L domain of 3G4, the reverse primer V_L-GGSSRSS-Rev contained the

underlined nucleotides encoding a hydrophobic short linker (GGSSRSS), which was used for linking 3G4 V_L domain to 3G4 V_H domain. The CD6-3G4 V_L domain was amplified by PCR using pCMV-(CD6-3G4$_m$-FLAG) as template and primer pairs of SmaI-CD6-Fwd / V_L-GGSSRSS-Rev. Briefly, 100ng pCMV-(CD6-3G4$_m$-FLAG) and 2.5µL of each primer (10µM) were added into 25µL Phusion High-Fidelity PCR Master Mix in a PCR tube and the final volume was adjusted to 50µL with MQ-H_2O. All amplification reactions were run in a thermal cycler with the following program: initial denaturation at 98^{o}C for 30 seconds, 40 cycles composed of denaturation at 98^{o}C for 15 seconds, annealing at 55^{o}C for 15 seconds, and extension at 72^{o}C for 45 seconds, and was ended with a final extension step of 10 min at 72^{o}C. PCR product with the expected size (498bp) was agarose gel purified.

For cloning the V_H domain of 3G4, the forward primer GGSSRSS-V_H-Fwd contained the underlined nucleotides coding for a hydrophobic short linker (GGSSRSS), which was used for linking the 3G4 V_H domain to 3G4 V_L domain. The reverse primer V_H-$(G_4S)_3$-Rev contained the underlined nucleotides coding for $(G_4S)_3$, which was used for linking CD6-3G4$_d$ to Cpα_{CD}-FLAG. The 3G4 V_H domain was amplified by PCR using pCMV-(CD6-3G4$_m$-FLAG) as template and primer pairs of GGSSRSS-V_H-Fwd / V_H-$(G_4S)_3$-Rev. Briefly, 100ng pCMV-(CD6-3G4$_m$-FLAG) and 2.5µL of each primer (10µM) were added into 25µL Phusion High-Fidelity PCR Master Mix in a PCR tube and the final volume was adjusted to 50µL with MQ-H_2O. The amplification reaction was run in a thermal cycler with the following program: initial denaturation at 98^{o}C for 30 seconds, 40 cycles consisting of denaturation at 98^{o}C for 15 seconds, annealing at 55^{o}C for 15 seconds, and extension at 72^{o}C for 45 seconds, and was ended with a final extension step of 10 min at 72^{o}C. PCR product with the expected size (387bp) was agarose gel purified.

CD6-3G4-V_L-GGSSRSS and GGSSRSS-3G4-V_H-$(G_4S)_3$ were joined using the GGSSRSS linker in an assembly reaction. When the GGSSRSS of CD6-3G4-V_L-GGSSRSS anneals to the GGSSRSS of GGSSRSS-3G4-V_H-$(G_4S)_3$, a fill-in reaction is primed in the presence of Phusion High-Fidelity DNA Polymerase. For this reaction to proceed efficiently, approximately equal molar quantities of CD6-3G4-V_L-GGSSRSS and GGSSRSS-3G4-V_H-$(G_4S)_3$ were added into 25μL Phusion High-Fidelity PCR Master Mix in a PCR tube, and the tube was placed in a thermocycler with the following program: initial denaturation at 98°C for 30 seconds, 10 cycles composed of denaturation at 98°C for 1 minutes, annealing at 50°C for 15 seconds, and extension at 72°C for 45 seconds, and was ended with a final extension step of 10 min at 72°C. In the second round of PCR, the assembled CD6-3G4$_d$-$(G_4S)_3$ was amplified by PCR using assembled and filled-in PCR product from the previous step as template and primer pairs of SmaI-CD6-Fwd and V_H-$(G_4S)_3$-Rev. Briefly, 100ng assembled and filled-in CD6-3G4$_d$-(G_4S) and 2.5μL of each primer (10μM) were added into 25μL Phusion High-Fidelity PCR Master Mix (New England Biolabs) in a PCR tube, and the final volume in the tube was adjusted to 50μL with MQ-H_2O. The amplification reaction was run in a thermal cycler with the following program: initial denaturation at 98°C for 30 seconds, 40 cycles composed of denaturation at 98°C for 15 seconds, annealing at 55°C for 15 seconds, and extension at 72°C for 45 seconds, and was ended with a final extension step of 10 min at 72°C.

PCR product with the expected size (840bp) was agarose gel purified and cloned into pcr4-TOPO (Invitrogen), and the DNA sequence of CD6-3G4$_d$-$(G_4S)_3$ was confirmed by DNA sequencing. Positive clones were used as template for amplifying CD6-3G4$_d$-$(G_4S)_3$ with the CD6-3G4$_d$-Cpα_{CD}-FLAG chimera as a negative control.

Table 3: Primers for converting 3G4 scFv monomer to diabody	
Primers	Sequence (5'→3')
SmaI-CD6-Fwd	CCCGGGACCATGCCCATGGGGTCTCTGCAA
V_L-GGSSRSS-Rev	GGAAGATCTAGAGGAACCACCTGAGGAGACGGTGACCGTGGTCCCT
GGSSRSS-V_H-Fwd	GGTGGTTCCTCTAGATCTTCCGACATCCAGCTCACTCAGTCTCCAG
V_H-$(G_4S)_3$-Rev	CCGCCAGAGCCACCTCCGCCTGAACCGCCTCCACCTTTCAGCTCCAGCTTGGTCCCCGAG

Preparation of C. perfringens genomic DNA

C. perfringens strain NRRL B-23706 (Southern Plains Agricultural Research Center, College Station, TX) was grown in PYG medium [2% (w/v) peptone, 0.5% (w/v) glucose, 1% (w/v) yeast extract in MQ-H_2O supplemented with 2mM NaCl, 3mM cysteine hydrochloride, 80μM $CaCl_2$, 65μM $MgSO_4$, 0.3mM K_2HPO_4, 0.3mM KH_2PO_4, and 5mM $NaHCO_3$; pH was adjusted to 7.2] in an atmosphere of 5% CO_2, 5% H_2, and 90% N_2 overnight. Genomic DNA of *C. perfringens* was purified using MasterPure™ Gram Positive DNA Purification Kit (Epicentre® Biotechnologies, Madison, Wisconsin). One mL of an overnight *C. perfringens* culture was pelleted by centrifugation. The bacterial pellet was resuspended in 150μL Tris-EDTA buffer (TE buffer). One μL Ready-Lyse lysozyme was added into the bacterial suspension, and the lysis reaction of the bacterial cell wall was allowed to proceed for 30 minutes at 37°C. For maximum bacterial cell wall lysis, bacterial crude lysate was further mixed thoroughly with 150μL Proteinase K / Gram Positive Lysis Solution and incubated for 15 minutes at 65°C. Bacterial debris was precipitated by adding 175μL MPC Protein Precipitation Reagent and removed by centrifugation. Trace RNA was removed by addition of 1μL RNase A (5μg/μL) (SIGMA) and incubation for 30 minutes at

37°C. Genomic DNA was precipitated by addition of 500µL isopropanol with end-over-end mixing for 10 minutes at room temperature. Genomic DNA was then pelleted by centrifugation and washed with 70% ethanol three times. The genomic DNA pellet was air-dried in a laminar flow hood for 30 minutes and then resuspended in 50µL TE buffer. Concentration of genomic DNA was determined on the NanoDrop.

Cloning cDNA encoding $(G_4S)_3$-Cpα$_{CD}$-FLAG

Primers used to amplify the gene encoding $(G_4S)_3$-Cpα$_{CD}$-FLAG were listed in Table 4. The forward primer $(G4S)_3$-Cpα$_{CD}$-Fwd contained the underlined nucleotides encoding $(G_4S)_3$, which was used for linking the Cpα$_{CD}$ gene to DAG1 / 3G4$_d$. The reverse primer Cpα$_{CD}$-FLAG-Rev contained the underlined nucleotides introducing a FLAG tag (DYKDDDDK) at the 3' end, which was used for detecting complete protein expression of CD6-DAG1-Cpα$_{CD}$-FLAG / CD6-3G4$_d$-Cpα$_{CD}$-FLAG. The $(G4S)_3$-Cpα$_{CD}$-FLAG was amplified by PCR using *C. perfringens* genomic DNA as template and primer pairs of $(G4S)_3$-Cpα$_{CD}$-Fwd and Cpα$_{CD}$-FLAG-Rev. Briefly, 100ng *C. perfringens* genomic DNA and 2.5µL of each primer (10µM) were added into 25µL Phusion High-Fidelity PCR Master Mix (New England Biolabs) in a PCR tube, and final volume in the tube was adjusted to 50µL with MQ-H_2O. The amplification reaction was run in a thermal cycler with the following program: initial denaturation at 98^{o}C for 30 seconds, 40 cycles composed of denaturation at 98^{o}C for 15 seconds, annealing at 55^{o}C for 15 seconds, and extension at 72^{o}C for 45 seconds, and was ended with a final extension step of 10 min at 72^{o}C. PCR product with the expected size (618bp) was agarose gel purified and cloned into pcr4-TOPO vector (Invitrogen), and DNA sequence of $(G_4S)_3$-Cpα$_{CD}$-FLAG was confirmed by sequencing. Positive clones were

used as template for amplifying $(G_4S)_3$-Cpα_{CD}-FLAG for CD6-DAG1-Cpα_{CD}-FLAG and CD6-3G4$_d$-Cpα_{CD}-FLAG cloning.

Table 4: Primers for cloning $(G_4S)_3$-Cpα_{CD}-FLAG	
Primers	Sequence (5'→3')
$(G_4S)_3$-Cpα_{CD}-Fwd	GTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCAT CAAAAGAATATGCAAGAGGTTTTG
Cpα_{CD}-FLAG-Rev	TGGATCCTACTTGTCATCGTCATCCTTGTAATCTTTTA TATTATAAGTTGAATTTCCT

Cloning of CD6-DAG1-Cpα_{CD}-FLAG / CD6-3G4$_d$-Cpα_{CD}-FLAG

Primers used to amplify the gene coding for CD6-DAG1-Cpα_{CD}-FLAG / CD6-3G4$_d$-Cpα_{CD}-FLAG were listed in Table 5. CD6-DAG1-$(G_4S)_3$ / CD6-3G4$_d$-$(G_4S)_3$ and $(G_4S)_3$-Cpα_{CD}-FLAG were joined into a single chain by attachment of the $(G_4S)_3$ linker in an assembly reaction. When the $(G_4S)_3$ of CD6-DAG1-$(G_4S)_3$ / CD6-3G4$_d$-$(G_4S)_3$ anneals to the $(G_4S)_3$ of $(G_4S)_3$-Cpα_{CD}-FLAG, they prime a fill-in reaction in the presence of Phusion High-Fidelity DNA Polymerase. For this reaction to proceed efficiently, approximately equal molar quantities of CD6-DAG1-$(G_4S)_3$ / CD6-3G4$_d$-$(G_4S)_3$ and $(G_4S)_3$-Cpα_{CD}-FLAG were added into 25μL Phusion High-Fidelity PCR Master Mix (New England Biolabs) in a PCR tube, and the tube was placed in a thermocycler with the following program: initial denaturation at 98^{o}C for 30 seconds, 10 cycles composed of denaturation at 98^{o}C for 1 minutes, annealing at 50^{o}C for 15 seconds, and extension at 72^{o}C for 45 seconds, and was ended with a final extension step of 10 min at 72^{o}C. In the second round of PCR, the assembled CD6-DAG1-Cpα_{CD}-FLAG / CD6-3G4$_d$-Cpα_{CD}-FLAG was amplified by PCR using assembled and filled-in PCR product from the previous step as template and SmaI-

CD6-Fwd and Cpα$_{CD}$-FLAG-Rev primer pairs. Briefly, 100ng assembled and filled-in PCR product and 2.5μL of each primer (10μM) were added into 25μL Phusion High-Fidelity PCR Master Mix (New England Biolabs) in a PCR tube, and final volume in the tube was adjusted to 50μL with MQ-H_2O. The amplification reaction was run in a thermal cycler with the following program: initial denaturation at 98^{o}C for 30 seconds, followed by 40 cycles composed of denaturation at 98^{o}C for 15 seconds, annealing at 55^{o}C for 15 seconds, and extension at 72^{o}C for 45 seconds, and was ended with a final extension step of 10 min at 72^{o}C. PCR products with the expected size (CD6-DAG1-Cpα$_{CD}$-FLAG: 1506bp; CD6-3G4$_d$-Cpα$_{CD}$-FLAG: 1410bp) were agarose gel purified and then cloned into pcr4-TOPO (Invitrogen), and DNA sequence of CD6-DAG1-Cpα$_{CD}$-FLAG / CD6-3G4$_d$-Cpα$_{CD}$-FLAG was confirmed by DNA sequencing. Positive clones were used as template for amplifying CD6-DAG1-Cpα$_{CD}$-FLAG / CD6-3G4$_d$-Cpα$_{CD}$-FLAG, to be further used for adenovirus vector construction.

Table 5: Primers for cloning CD6-DAG1-Cpα$_{CD}$-FLAG / CD6-3G4$_d$-Cpα$_{CD}$-FLAG

Primers	Sequence (5'→3')
SmaI-CD6-Fwd	CCCGGGACCATGCCCATGGGGTCTCTGCAA
Cpα$_{CD}$-FLAG-Rev	TGGATCCTACTTGTCATCGTCATCCTTGTAATCTTTTA TATTATAAGTTGAATTTCCT

Cloning of pDONR-(CD6-DAG1-Cpα$_{CD}$-FLAG) / pDONR-(CD6-3G4$_d$-Cpα$_{CD}$-FLAG)

PCR was used to efficiently produce *att*B1- and *att*B2-flanked CD6-DAG1-Cpα$_{CD}$-FLAG / CD6-3G4$_d$-Cpα$_{CD}$-FLAG open reading frame (ORF) fragments. Universal *att*B1 and *att*B2 adaptor primers (Table 6) with two chimeric sequences were used to integrate

complete *att*B1 and *att*B2 sites into the ORF amplicon. Phusion® High-Fidelity DNA Polymerase was employed to amplify the predicted ORF with high fidelity. The amplification was performed, according to the protocol from Invitrogen in a 50µL reaction mixture containing 10µL of 5x Phusion HF Reaction Buffer, 1µL of dNTPs (10mM), 2.5µL of each primer (10µM), 0.5µL of Phusion DNA polymerase and 1µL of template. The *att*B x *att*P (BP) recombination reaction directed by BP clonase was carried out between the PCR product and destination vector (pDONR™ 221) according to the Invitrogen's protocols (Invitrogen). Briefly, PCR product of right size was agarose gel purified and mixed with 150ng pDONR™ 221 vector in a 1.5mL microcentrifuge tube at room temperature. BP Clonase™ II enzyme mix (Invitrogen) was thawed on ice for two minutes, and then 2µL BP Clonase™ II enzyme mix was added to the reactant from the previous step. BP recombination reaction was allowed to proceed at room temperature for one hour. The reaction was terminated by adding 1µL Proteinase K solution (Invitrogen), followed by 10-minute incubation at 37°C. One µL of each BP recombination reactant was used for transformation into 50µL One Shot® OmniMAX™ 2 T1 phage-resistant chemically competent cells (Invitrogen). Two hundred and fifty µL S.O.C. medium was added to the cells, which were incubated at 37°C for one hour with shaking. One hundred µL of each transformation was plated onto LB plate [1% tryptone (w/v), 0.5% yeast extract (w/v), 1% NaCl (w/v), and 1.5% agar (w/v) in MQ-water] containing Kanamycin (50µg/mL). Five colonies were picked up and cultured overnight in LB medium [1% tryptone (w/v), 0.5% yeast extract (w/v), 1% NaCl (w/v)] containing Kanamycin (50µg/mL). The next day, plasmid DNA was isolated using the PureLink™ HQ Mini Plasmid Purification Kit (Invitrogen). All plasmid DNA was analyzed by restriction digestion and PCR to confirm the

presence and correct orientation of the inserted gene. Positive clones were further confirmed by DNA sequencing. CD6-DAG1-Cpα_{CD}-FLAG) and CD6-3G4$_d$-Cpα_{CD}-FLAG on pDONR was further cloned into pAd/CMV/V5-DEST™ vector (Invitrogen) separately by *att*L x *att*R (LR) recombination reaction.

Table 6: Primers for cloning *att*B1-(CD6-DAG1-Cpα_{CD}-FLAG)-*att*B2 and *att*B1-(CD6-3G4$_d$-Cpα_{CD}-FLAG)-*att*B2

Primers	Sequence (5'→3')
***att*B1 Fwd**	GGGGACAAGTTTGTAC**AAAAAA**GCAGGCTTCATGCC CATGGGGTCTCTGCAA
***att*B2 Rev**	GGGGACCAC**TTTGTA**CAAGAAAGCTGGGTCCTACTT GTCATCGTCATCCTTGTAATC

Cloning of pAd-(CD6-DAG1-Cpα_{CD}-FLAG) / pAd-(CD6-3G4$_d$-Cpα_{CD}-FLAG)

One hundred and fifty ng pDONR-(CD6-DAG1-Cpα_{CD}-FLAG) / pDONR-(CD6-3G4$_d$-Cpα_{CD}-FLAG) was mixed with 150ng pAd/CMV/V5-DEST™ vector in a 1.5mL microcentrifuge tube at room temperature. LR Clonase™ II enzyme mix (Invitrogen) was thawed on ice for two minutes, and then 2µL LR Clonase™ II enzyme mix was added to the reactant from the previous step. LR recombination reaction was allowed to proceed at room temperature for one hour. The reaction was terminated by adding 1µL proteinase K solution (Invitrogen), and then incubated at 37°C for 10 minutes. One µL of each LR reaction was used for transformation of 50µL One Shot® TOP10 Competent Cells (Invitrogen). Briefly, cells and DNA were incubated on ice for 30 minutes; cells were then heat-shocked by incubating at 42°C for 30 seconds. Two hundred and fifty µL S.O.C. medium was added to the cells, which were then incubated at 37°C for one hour with shaking. One hundred µL of

each transformation reaction was plated onto LB plate containing carbenicillin (100µg/mL). Five colonies were picked up and cultured overnight in LB medium containing carbenicillin (100µg/mL). The next day, plasmid DNA was isolated using the PureLink™ HiPure Plasmid Miniprep Kit (Invitrogen). DNA sequencing was used to confirm the correct orientation of positive clones. The most promising clones were propagated, amplified, and maintained in the One Shot® *ccd*B Survival™ 2 T1R chemically competent *E. coli*.

Expression of CD6-DAG1-Cpα$_{CD}$-FLAG / CD6-3G4$_d$-Cpα$_{CD}$-FLAG in HEK 293A cells

One day before transfection, 5 x 10^5 HEK 293A cells per well were seeded in a 6-well plate. Cells were supplemented with 2mL normal growth medium [10% (v/v) FBS, 1% (v/v) penicillin/streptomycin, 0.1mM MEM non-essential amino acids, and 2mM L-glutamine in DMEM] in each well. The next day, culture medium from HEK 293A cells was removed and replaced with 1.5mL normal OptiMEM® I Medium (Invitrogen) containing 10% FBS. For preparing DNA-Lipofectamine™ 2000 complex for transfection, 1µg pAd-(CD6-DAG1-Cpα$_{CD}$-FLAG) / pAd-(CD6-3G4$_d$-Cpα$_{CD}$-FLAG) was diluted in 250µL Opti-MEM® I Medium, and 3µL Lipofectamine™ 2000 was also diluted in 250µL Opti-MEM® I Medium. After the 5 minute incubation at room temperature, diluted DNA and Lipofectamine™ 2000 were combined by gentle mixing. The mixture was incubated for 20 minutes at room temperature to allow the DNA-Lipofectamine™ 2000 complexes to form. DNA-Lipofectamine™ 2000 complex was added drop wise to each well, and mixed gently by rocking the plate back and forth. Cells were incubated overnight at 37°C in a CO_2 incubator. The next day, the medium containing the DNA-Lipofectamine™ 2000 complex was removed and replaced with complete culture medium. Forty eight hours post-transfection, cells were

fixed with 500µL cold methanol in each well for 1 minute and blocked with PBST containing 5% (w/v) BSA overnight at 4°C. Cells were then incubated with alkaline phosphatase conjugated anti-FLAG mAb (SIGMA) diluted (1:1000) in PBST containing 3% (w/v) BSA for one hour at room temperature. The color reaction was developed by using SIGMA*FAST*™ Fast Red TR/Naphthol AS-MX substrate (SIGMA) per the manufacturer's instructions, and terminated by addition of 0.1M EDTA. Specific staining was visualized using an inverted phase contrast tissue culture microscope.

Production of recombinant adenovirus expressing CD6-DAG1-Cpα$_{CD}$-FLAG / CD6-3G4$_d$-Cpα$_{CD}$-FLAG

One day before transfection, HEK 293A cells were seeded at 5x10^5 cells per well in a 6-well plate. Cells were plated in 2mL complete culture medium. On the next day, culture medium was removed from cells and replaced with 1.5mL Opti-MEM® I Medium containing 10% FBS. In order to completely expose the inverted terminal repeats (ITRs) (adenoviral late genes encoding the encapsidation signal) on adenoviral vectors, both pAd-(CD6-DAG1-Cpα$_{CD}$-FLAG) and pAd-(CD6-3G4$_d$-Cpα$_{CD}$-FLAG) were linearized by *Pac*I digestion before transfection of HEK 293A cells. Digested DNA was purified using phenol/chloroform extraction followed by ethanol precipitation. Linear DNA was resuspended in MQ-water to a final concentration of 500ng/µL. To prepare DNA-Lipofectamine™ 2000 complexes for transfecting HEK 293A cells, 2µg DNA was diluted in 250µL Opti-MEM® I Medium, and 5µL Lipofectamine™ 2000 was gently mixed in 250 µL Opti-MEM® I Medium as well. After a 5-minute incubation at room temperature, diluted DNA and diluted Lipofectamine™ 2000 were combined, mixed gently, and incubated for 20 minutes at room temperature to

allow the DNA-Lipofectamine™ 2000 complexes to form. HEK 293A cells were transfected by adding the DNA-Lipofectamine™ 2000 complexes drop wise to each well, then the plate was rocked back and forth gently to enhance transfection efficacy. Transfected cells were incubated at 37°C in a CO_2 incubator overnight. The next day, the medium containing the DNA-Lipofectamine™ 2000 complexes was removed and replaced with complete culture medium. Forty eight hours post-transfection, transfected cells were trypsinized and contents of each well was transferred to a sterile 10cm cell culture petri dish. Cell culture medium was replaced with fresh, complete culture medium every 3 days. Twelve days post-transfection (around cytopathic effects were observed in 80% of the cells), adenovirus-containing cells were harvested by squirting cells off the plate with a 10mL serological pipette. Cells and adenovirus-containing medium were transferred to a sterile 15 mL conical tube.

Preparation of adenovirus stock

The tube containing harvested adenovirus-containing cells and medium was placed at -80^{o}C for 30 minutes. The tube was removed from -80^{o}C and placed in a 37^{o}C water bath for 15 minutes to thaw. The freezing and thawing steps were repeated twice. Cell lysate was centrifuged at 3000 rpm for 15 minutes at room temperature to pellet the cell debris. The supernatant containing adenoviral particles was transferred to cryovials in 1mL aliquots. Adenovirus stocks were stored at -80°C.

Amplification of adenovirus

Three days before infection, HEK 293A cells were seeded at 3 x 10^6 cells per T175 cell culture flask with complete culture medium. On the day of infection, cell density was

verified to be at 80-90% confluence before proceeding. One hundred μL adenovirus stock was added to the cells, and the cell culture flask was swirled gently to maximize infection efficiency. Cells were incubated in a CO_2 incubator at 37°C and infection was allowed to proceed until 80-90% of the cells had rounded up and were floating or lightly attached to the tissue culture dish. Adenovirus-containing cells were harvested by squirting cells off from the flask with a sterile 10mL serological pipet. Cells and supernatant were transferred to a sterile 50mL conical centrifuge tube. After centrifugation at 3000rpm for 15 minutes, supernatant was removed and cell pellets were placed at -80°C for 30 minutes. Frozen cells were then incubated in a 37°C water bath for 15 minutes to thaw. Freezing and thawing steps were repeated twice. Cell lysate was centrifuged at 3000rpm for 15 minutes at room temperature to pellet the cell debris. Supernatant containing viral particles was transferred to cryovials in 1mL aliquots. Amplified adenoviruses were stored at -80°C and proceed to viral tittering.

Titration of adenovirus stock

A particular challenge in the delivery of a gene by adenoviral vector is the accurate measurement of virus titer. QuickTiter™ Adenovirus Titer Immunoassay Kit (Cell Biolabs, San Diego, CA), which utilizes an antibody against adenovirus hexon proteins to visualize infected cells by immunocytochemical staining, was used to functionally titer infectivity of both Ad/CD6-DAG1-Cpα_{CD}-FLAG and Ad/CD6-3G4$_d$-Cpα_{CD}-FLAG. Two and a half x 10^5 HEK 293A cells were seeded in each well of a 24-well cell culture plate, and then incubated at 37°C with supplement of 5% CO_2 for one hour. Immediately before infection, 10-fold serial dilutions of Ad/CD6-DAG1-Cpα_{CD}-FLAG / Ad/CD6-3G4$_d$-Cpα_{CD}-FLAG ranging from

10^{-5} to 10^{-9} were prepared. One hundred μL of each diluted adenovirus sample was added to each well of this 24-well cell culture plate. Titrations were performed in triplicate to ensure accuracy. Infected HEK 293 cells were incubated at 37°C with supplement of 5% CO_2 for two days. Forth eight hours post infection, infected HEK 293A cells were fixed by gently adding 0.5mL cold methanol down the side of each well of the 24-well cell culture plate. Cells were gently washed three times with PBST, and then blocked with PBST containing 5% BSA at 4°C overnight. On the next day, 1X anti-hexon antibody solution was prepared by diluting 1000X stock in PBST containing 3% BSA. Two hundred fifty μL 1X anti-hexon antibody solution was added to each well. The plates were incubated at room temperature on an orbital shaker for one hour. Cells were gently washed three times with PBST. In the meantime, 1X secondary antibody (HRP-conjugated) solution was prepared by diluting 1000X stock in PBST containing 3% BSA. Two hundred and fifty μL secondary antibody solution was added to each well, and the plate was incubated at room temperature on an orbital shaker for one hour. Cells were gently washed three times with PBST. DAB working solution prepared per manufacture's manual for color development on positive cells. Two hundred and fifty μL freshly prepared DAB working solution was added to each well, and then the plate was incubated at room temperature on an orbital shaker for 10 minutes. Stained cells were counted in at least five separate fields per well using a light microscope and the 10X objective. Average number of positive cells was counted per well, and viral titer [infectious units (ifu) / mL] was calculated by the formula below.

$$\text{Viral titer (ifu/mL)} = \frac{(\text{average positive cells/field}) \times (79 \text{ fields/well}) \times (\text{dilution factor})}{(0.1\text{mL})}$$

In vitro expression of DAG1-Cpα$_{CD}$-FLAG and 3G4$_d$-Cpα$_{CD}$-FLAG and assessment of their specific binding to chCD40

HEK 293A cells were seeded in a 10cm cell culture petri dish at a density of about 2.5×10^5 cells/cm^2 in complete medium. After 3 days of confluence, cells were infected with Ad-(CD6-DAG1-Cpα$_{CD}$-FLAG) /Ad-(CD6-3G4$_d$-Cpα$_{CD}$-FLAG) at a multiplicity of infection (MOI) of 1. Seventy-two hours post infection; 10mL cell culture supernatant was harvested and concentrated to 500µL using Pierce Protein Concentrator, 9K MWCO (Thermo Scientific). Specific binding of CD6-DAG1-Cpα$_{CD}$-FLAG and CD6-3G4$_d$-Cpα$_{CD}$-FLAG to chCD40 were accessed by sandwich ELISA. Briefly, ELISA plates were coated with rchCD40$_{ED}$ and then incubated with concentrated DAG1-Cpα$_{CD}$-FLAG / 3G4$_d$-Cpα$_{CD}$-FLAG from cell culture medium. Specific binding of DAG1-Cpα$_{CD}$-FLAG / 3G4$_d$-Cpα$_{CD}$-FLAG was then detected by incubating with chicken serum against Cpα followed by horseradish peroxidase (HRP) conjugated rabbit anti-chicken IgG antibody (Thermo Scientific).

Vaccination and challenge

Since recombinant adenovirus in this study not only produces the Cpα$_{CD}$ but the Cpα$_{CD}$ fused with a CD40-targeting diabody, we hypothesized that substantially lower viral doses ($3x10^8$ to $3x10^9$ ifu) would be sufficient to elicit proactive humoral responses. Cobb 500 embryonated eggs of 18 days (E18) were used for all *in ovo* treatments. Based on the visibility of the embryo, a small hole was made through the air cell with a drill. *In ovo* vaccination of 45 embryonated eggs was completed by inoculation of Ad-(CD6-DAG1-Cpα$_{CD}$-FLAG) / Ad-(CD6-3G4d-Cpα$_{CD}$-FLAG) from $3x10^8$ to $3x10^9$ ifu/dose diluted in 300µL sterile PBS respectively. Recombinant adenovirus was injected into the allantoic

cavity of eggs (at a depth of one inch) using a 21-gauge needle. In addition, 45 eggs were *in ovo* inoculated with 300μL saline, 45 eggs with phospholipase C from *C. perfringens* (SIGMA) mixed with modified chitosan, and 45 eggs with Cpα peptide-KLH conjugate mixed with modified chitosan for controls. Vaccinated eggs were incubated at 37°C in an egg incubator until they hatched. Hatched chickens were maintained in floor pen in a biosafety level 2 climate-controlled room and provided with water and 55% wheat/corn-based broiler starter diet *ad libitum.* Modified chitosan is produced by deacetylation of chitin from the shells of shrimp and other sea crustaceans (Wang et al., 2011). Modified chitosan has been used as adjuvant for poultry vaccine for a long time, and the positive adjuvant effect of chitosan on antigen-specific cell-mediated immunity after chickens vaccination with live Newcastle disease vaccine was also reported (Rauw et al., 2010). On day 14 post hatch, chickens were starved for 24 hours before 30,000 oocysts of *Eimeria maxima* were administered orally to each chicken by gavage. In the meantime, two virulent *C. perfringens* strains (Cp641 and Texas A&M) were grown in cooked meat medium (CMM) for 24 hours at 37^{o}C. Liquid thioglycolate medium was then inoculated with 3% (v/w) inoculum and then incubated at 37^{o}C for 24 hours. The growth of *C. perfringens* at 24 hours was approximately 10^{8} colony-forming units per mL (CFUs). Four days post administration with *Eimeria maxima*, feed was withdrawn and each bird was orally challenged with 1.5mL *C. perfringens* Cp641 (10^{8} CFU) and Texas A&M (10^{8} CFU) strains. All experimental procedures and animal care were performed in compliance with all applicable federal and institutional animal use guidelines. The conditions for animal use here were approved by the Institutional Animal Care & Use Committee of the University of Arkansas.

Necropsy and weight gain

Four days post *C. perfringens* challenge, all chickens were euthanized by CO_2 asphyxiation and all birds were then weighed individually. Their small intestines (duodenum to ileum) were examined for grossly visible lesions. Intestinal lesions were scored from 0 to 4, where 0 = no gross lesions; 1 = thin and friable wall; 2 = focal necrosis or ulceration (1-5 foci); 3 = focal necrosis or ulceration (6-15 foci); 4 = diffuse necrotic typical of field cases. Blinding was employed to avoid scorer bias.

Quantification of Cpα-specific serum IgG and tracheal IgA by ELISA

The Cpα-specific immune responses were monitored by collecting serum and tracheal mucosal samples on day 4, day 11 post hatches, and 4 days post *C. perfringens* challenge. ELISA was used for quantification of Cpα-specific IgG and IgA antibody titers.

For collecting mucosal IgA sample from chicken trachea, a 1-cm segment of trachea from each bird was collected in a 2mL microcentrifuge tube, and the trachea was suspended in cold PBS [1:1 (wt/vl)] containing 0.01M phenylmethylsulfonyl fluoride, 0.1% sodium azide, and 3% BSA. Mucosal IgA was sloughed off from the inner liner of trachea by vigorous vortexing for 30 seconds. The tube was centrifuged at 5000*g* for 30 min at 4°C, and the supernatant was collected and frozen at -20^{o}C until use.

Ninety-six-well-microtiter plates (Nunc) were coated with purified Cpα (United States Department of Agriculture, IA) diluted in coating buffer overnight at 4^{o}C. Unadsorbed Cpα was removed, and non-specific binding was blocked by incubating with PBST containing 5% BSA overnight at 4^{o}C. After blocking, coated plates were incubated with samples of diluted serum (1:100) / tracheal mucus suspension (1:5) in PBST containing 3% (w/v) BSA in

triplicate, as well as positive control (Cpα-specific chicken serum, 1:100) and negative control (normal chicken serum, 1:100) overnight at 4°C. After repeat washes with PBST, PBST diluted horseradish peroxidase conjugated rabbit anti-chicken IgY (H+L) (1:12,000) / horseradish peroxidase conjugated goat anti-chicken IgA (H+L) (1:3000) was added to the wells and incubated at room temperature for one hour. Plates were washed for several times with PBST, and the color reaction was developed using OptEIA™ TMB substrate (Beckton Dickinson) per the manufacturer's instructions, and terminated by addition of 50μL 1N sulfuric acid per well. Absorbance at 450 nm [A_{450}] was measured in a Wallac plate reader. The presence of Cpα-specific IgG / IgA was determined by relating the mean A(450) value of each serum sample / mucosal IgA to that of the chicken Cpα positive control serum. The relative levels of Cpα-specific IgG / IgA in all samples were determined and normalized by calculating the sample to positive (S/P) ratio as described in Chapter IV. Comparison of means of S/P values was performed using one-way analysis of variance (ANOVA) with least significant difference (L.S.D.) as multiple comparison tests. Student's t-test was used to determine significant differences in means of S/P values between treatments across all groups, and S/P values of the SA-peptide complex group were used as baseline. All data were analyzed and generated using SPSS software (IBM, Armonk, New York). Statistical significance was determined at $P < 0.05$.

Statistical analysis

Lesion scores were compared using two-tailed Fisher's exact test. Lesion scores were ranked from 0 to 4. A protective response was considered as birds with ≤ 1+ lesions. Body weight was analyzed using ANOVA.

Results and discussion

CD6-3G4-V_L (Fig. 12A) and 3G4-V_H-$(G_4S)_3$ (Fig. 12B) were joined into a single chain with a GGSSRSS linker in an assembly PCR reaction. PCR product with the expected size (840bp, Fig. 12C) was successfully amplified, gel purified, and then cloned into pcr4-TOPO vector. Positive clones were confirmed by DNA sequencing. Pcr4-[CD6-3G4$_d$-$(G_4S)_3$] was used as template for amplifying CD6-3G4$_d$-$(G_4S)_3$ for cloning of the CD6-3G4$_d$-Cpα_{CD}-FLAG chimera.

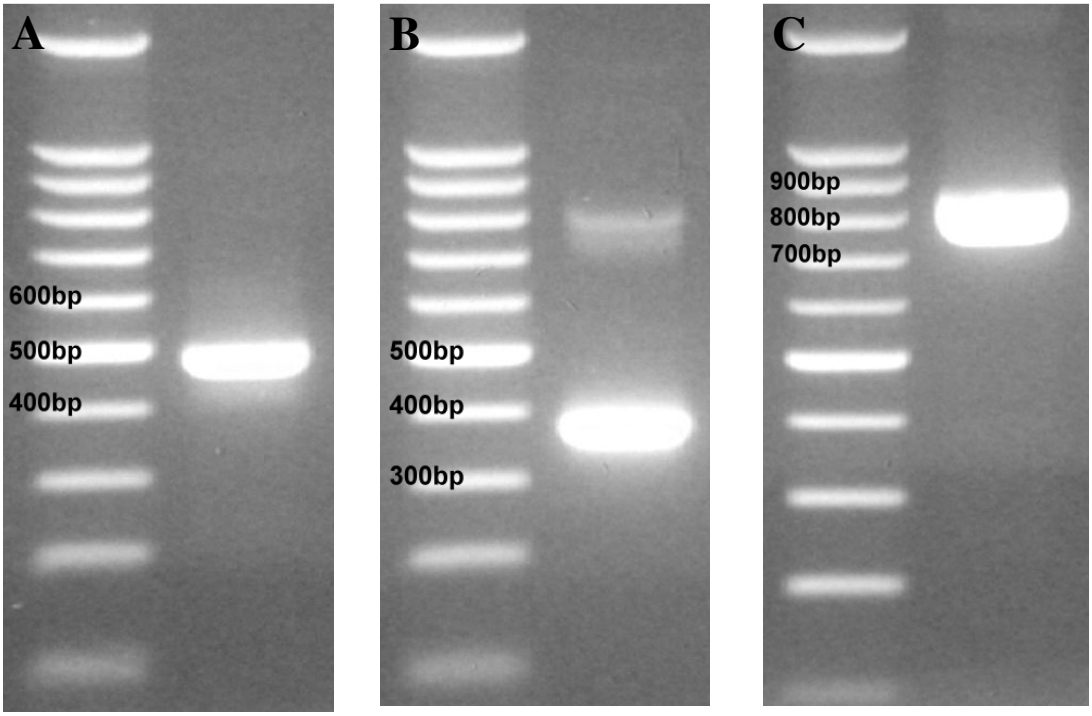

Fig. 12. Cloning of CD6-3G4$_d$-$(G_4S)_3$ from CD6-3G4 scFv monomer. (A) CD6-3G4-V_L: 498bp and (B) 3G4-V_H-$(G_4S)_3$: 387bp were amplified separately from CD6-3G4 scFv monomer. (C) CD6-3G4 scFv diabody with 3 x G_4S linker at its 3'-end: 840bp was prepared by linking CD6-3G4-V_L and 3G4-V_H-$(G_4S)_3$ with a short linker: GGSSRSS.

Cpα_{CD}-FLAG (Fig. 13B and 14B) and CD6-DAG1 (Fig. 13A) / CD6-3G4$_d$ (Fig. 14A) were joined together by a 3 x G_4S linker into a single chain by overlap PCR. PCR product with the expected size [CD6-DAG1-Cpα_{CD}-FLAG (1506bp) (Fig. 13C) and CD6-3G4$_d$-

Cpα_{CD}-FLAG (1410bp) (Fig. 14C) were successfully amplified, gel purified, and then cloned into pcr4-TOPO vector. Positive clones were confirmed by DNA sequencing. Pcr4-CD6-DAG1-Cpα_{CD}-FLAG / pcr4-CD6-3G4$_d$-Cpα_{CD}-FLAG was used as template for amplifying *att*B1-(CD6-DAG1-Cpα_{CD}-FLAG)-*att*B2 / *att*B1-(CD6-3G4$_d$-Cpα_{CD}-FLAG)-*att*B2 for a first round of homologous recombination in adenoviral vector preparation.

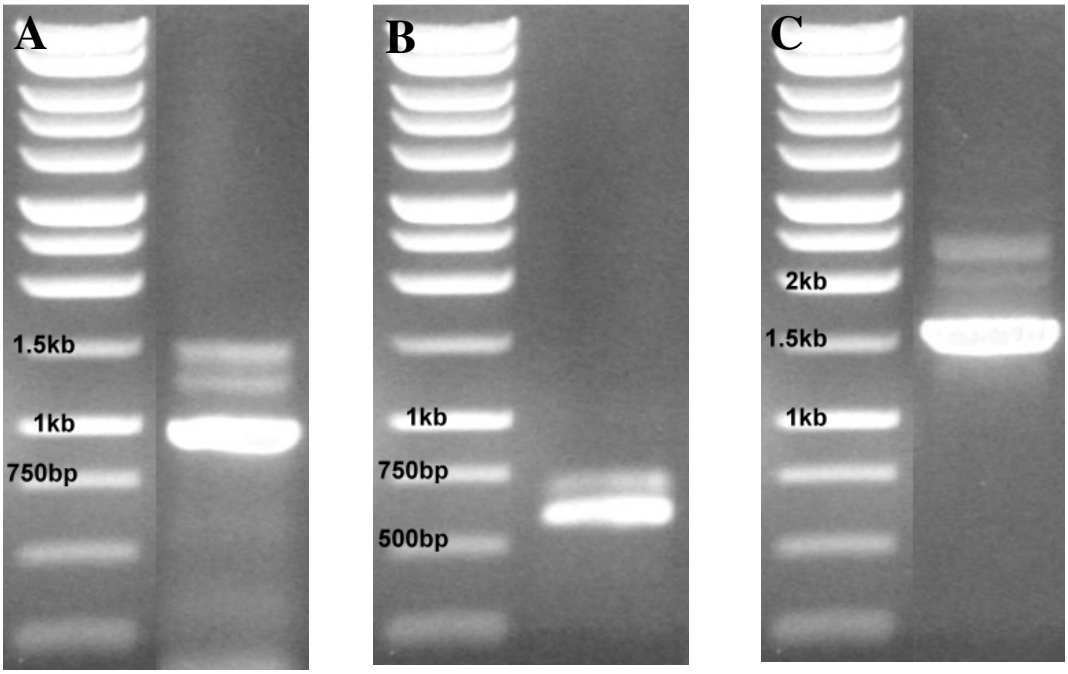

Fig. 13. Cloning of CD6-DAG1-Cpα_{CD}-FLAG by overlap extension PCR. (A) CD6-DAG1 scFv diabody with 3 x G_4S linker at its 3'-end: 933bp and (B) Cpα_{CD}-FLAG with 3 x G_4S linker at its 5'-end: 618bp were joined into a single chain, (C) CD6-DAG1-Cpα_{CD}-FLAG: 1506bp, by $(G_4S)_3$ linker in an assembly PCR reaction.

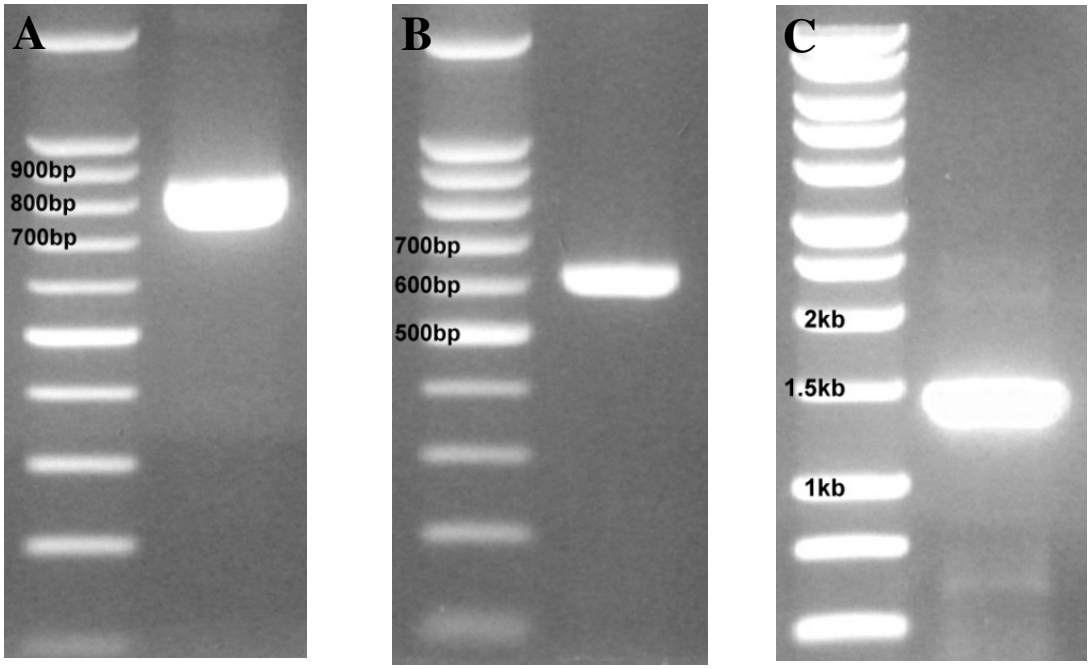

Fig. 14. Cloning of CD6-3G4$_d$-Cpα$_{CD}$-FLAG by overlap extension PCR. (A) CD6-3G4 scFv diabody with 3 x G$_4$S linker at its 3'-end: 837bp and (B) Cpα$_{CD}$-FLAG with 3 x G$_4$S linker at its 5'-end: 618bp were joined into a single chain, (C) CD6-3G4$_d$-Cpα$_{CD}$-FLAG: 1410bp, by (G$_4$S)$_3$ linker in an assembly PCR reaction.

*att*B1-(CD6-DAG1-Cpα$_{CD}$-FLAG)-*att*B2 (Fig. 15A) and *att*B1-(CD6-3G4$_d$-Cpα$_{CD}$-FLAG)-*att*B2 (Fig. 15B) were successfully amplified by PCR. The sizes of the amplicons were 1606bp and 1510bp, respectively, as expected in theory. CD6-DAG1-Cpα$_{CD}$-FLAG / CD6-3G4$_d$-Cpα$_{CD}$-FLAG in the pDONR® 221 vector was confirmed by PCR and DNA sequencing. CD6-DAG1-Cpα$_{CD}$-FLAG / CD6-3G4$_d$-Cpα$_{CD}$-FLAG in the pDONR® 221 vector was successfully transferred to pAd/CMV/V5-DEST™ vector by LR recombination. Positive pAd(CD6-DAG1-Cpα$_{CD}$-FLAG) / pAd-(CD6-3G4$_d$-Cpα$_{CD}$-FLAG) was purified and digested with *Pac* I enzyme. Two gene fragments with the length of 30 kb, and 2 kb were obtained and consistent with the expected fragment size. It is concluded that two recombinant adenovirus vectors were successfully constructed. Furthermore, two inserts in the recombinant adenovirus vector were both verified by DNA sequencing.

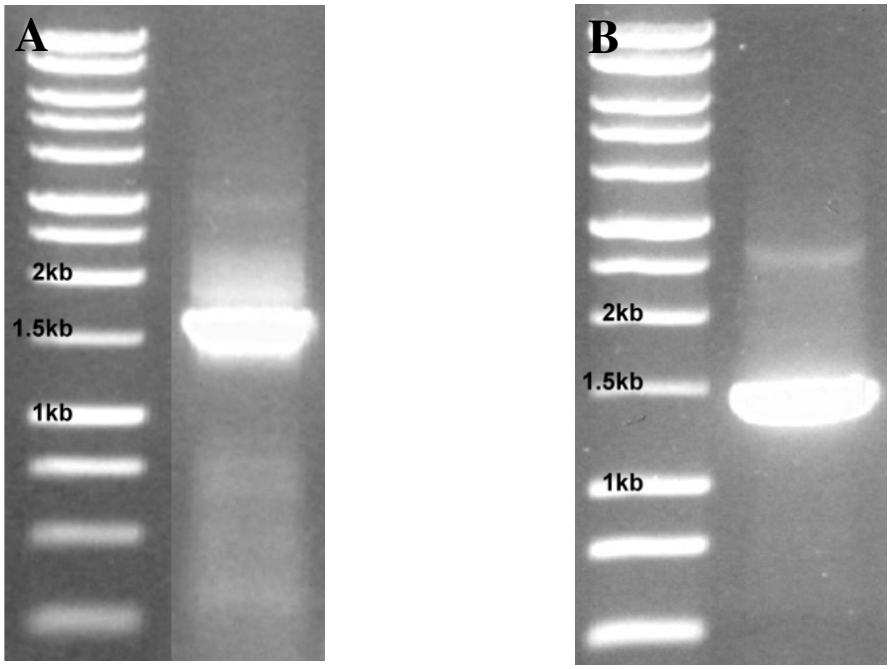

Fig. 15. Cloning of (A) *att*B1-(CD6-DAG1-Cpα$_{CD}$-FLAG)-*att*B2: 1576bp and (B) *att*B1-(CD6-3G4$_d$-Cpα$_{CD}$-FLAG)-*att*B2: 1480bp. PCR was used to efficiently produce *att*B1- and *att*B2-flanked CD6-DAG1-Cpα$_{CD}$-FLAG / CD6-3G4$_d$-Cpα$_{CD}$-FLAG ORF fragment. Universal *att*B1 and *att*B2 adaptor primers with two chimeric sequences were used to integrate complete *att*B1 and *att*B2 sites into the ORF amplicon.

To confirm that both chimeric proteins could be expressed, immunocytochemistry was used on HEK 293A cells transfected with pAd-(CD6-DAG1-Cpα$_{CD}$-FLAG) / pAd-(CD6-3G4$_d$-Cpα$_{CD}$-FLAG). Forty eight hours post-transfection, cells were fixed, blocked, and then stained with alkaline phosphatase conjugated anti-FLAG mAb**.** The specific recombinant chimeric protein DAG1-Cpα$_{CD}$-FLAG and 3G4d-Cpα$_{CD}$-FLAG expressed in HEK 293A cells was specifically detected by immunocytochemistry as shown in Fig. 16. Immunocytochemical staining clearly showed that the FLAG-positive cells were only observed in the transfected cells but not in normal HEK 293A cells (negative control data not shown).

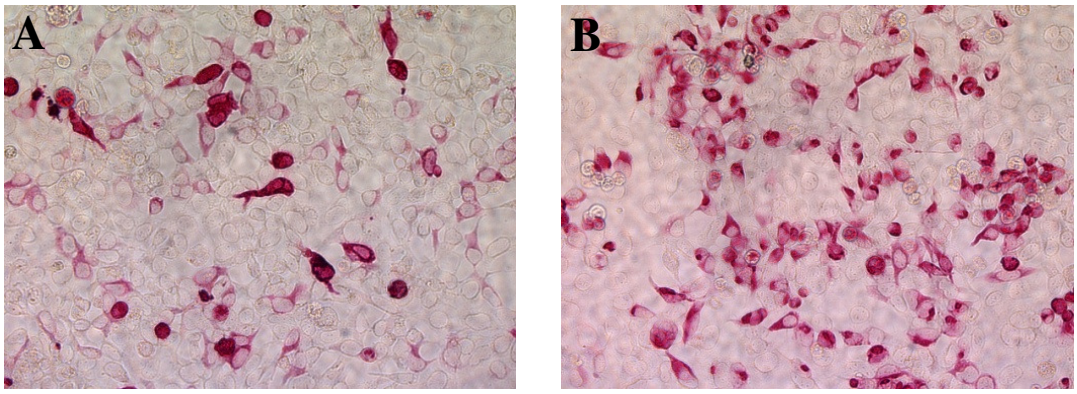

Fig. 16. Expression of CD6-DAG1-Cpα_{CD}-FLAG (A) and CD6-3G4$_d$-Cpα_{CD}-FLAG (B) in HEK 293A cells transfected with adenoviral vectors harboring the CD6-DAG1-Cpα_{CD}-FLAG / CD6-3G4$_d$-Cpα_{CD}-FLAG gene. Forty eight hours post-transfection, expression of DAG1-Cpα_{CD}-FLAG / 3G4$_d$-Cpα_{CD}-FLAG in transfected HEK 293A cells was visualized by red alkaline phosphatase (AP) staining (AP conjugated anti-FLAG mAb).

In order to allow proper viral replication and packaging in HEK 293A cells, left and right viral ITRs of pAd-(CD6-DAG1-Cpα_{CD}-FLAG) / pAd-(CD6-3G4$_d$-Cpα_{CD}-FLAG) need to be exposed by *Pac* I digestion before transfection of HEK 293A. Both pAd-(CD6-DAG1-Cpα_{CD}-FLAG) / pAd-(CD6-3G4$_d$-Cpα_{CD}-FLAG) were successfully linearized with *Pac* I (Fig. 17). Ampicillin resistance gene (2074bp) and the pUC origin on the vectors, which is useless in adenovirus production, were removed by *Pac* I digestion.

To determine whether host cells can express DAG1-Cpα_{CD}-FLAG or 3G4d-Cpα_{CD}-FLAG after infection with recombinant adenovirus, HEK 293 cells were exposed to various concentrations of either Ad-(CD6-DAG1-Cpα_{CD}-FLAG) or Ad-(CD6-3G4d-Cpα_{CD}-FLAG) for 48 hours. As shown in Fig. 18, two chimeric proteins were readily detected by immunocytochemistry in HEK 293 cells infected with 1 multiplicity of infection (MOI) of each recombinant adenovirus. Furthermore, the amount of each FLAG-positive chimeric protein increased with increasing MOI of its corresponding recombinant adenovirus (data not shown). There was no detectable FLAG in cells infected with sham adenovirus (data not

shown). The chCD40 binding capacity of DAG1-Cpα$_{CD}$-FLAG / 3G4d-Cpα$_{CD}$-FLAG chimeric protein was confirmed by ELISA (Table 7).

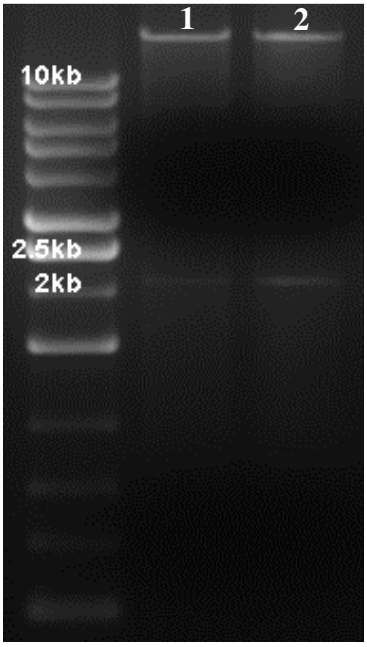

Fig. 17. Identification of recombinant pAd-(CD6-DAG1-Cpα$_{CD}$-FLAG) (lane1) and pAd-(CD6-3G4$_d$-Cpα$_{CD}$-FLAG) (lane 2) with *Pac* I digestion in 0.8% agarose gel. pUC origin and ampicillin resistance gene (2074bp) were released from both adenoviral vectors by *Pac* I digestion. Left and right viral ITRs of both adenoviral vectors were completely exposed for proper viral replication and packaging in HEK 293A cells.

Table 7: ELISA readouts [A(450)] indicated that only DAG1-Cpα$_{CD}$-FLAG has specific binding to chCD40 other than the control 3G4$_d$-Cpα$_{CD}$-FLAG				
	Concentrated DAG1-Cpα$_{CD}$-FLAG	**Concentrated 3G4$_d$-Cpα$_{CD}$-FLAG**	**Chicken anti-Cpα serum only**	**HRP-rabbit anti-chicken IgG antibody only**
A(450)	1.30	0.90	0.75	0.04
Chicken anti-Cpα serum (1:100, primary antibody) and HRP-rabbit anti-chicken IgG antibody (1:12000, secondary antibody) were used in this sandwich ELISA.				

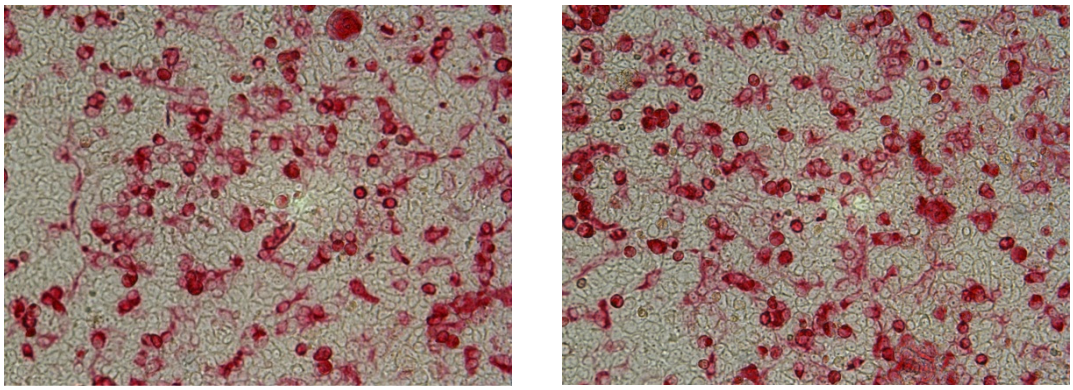

Fig. 18. Immunocytochemical staining of FLAG-tagged chimeric protein expression in Ad-(CD6-DAG1-Cpα$_{CD}$-FLAG) / Ad-(CD6-3G4$_d$-Cpα$_{CD}$-FLAG) infected HEK 293A cells. HEK 293A cells were infected with Ad-(CD6-DAG1-Cpα$_{CD}$-FLAG) (A) or Ad-(CD6-3G4$_d$-Cpα$_{CD}$-FLAG) (B) at MOI = 100 for 48 hours. After permeabilization, cells were incubated with AP-conjugated mouse anti-FLAG mAb, followed by color development using SIGMAFAST™ Fast Red TR/Naphthol AS-MX substrate.

From practical and commercial perspectives, *in ovo* vaccination has to maintain high hatchability levels (≥90%) (Josefsberg and Buckland, 2012). However, *in ovo* vaccination with Ad-(CD6-DAG1-Cpα$_{CD}$-FLAG) at 3x10^8 / 3x10^9 ifu on E18 eggs resulted in a poor hatchability (68-72%). On the other hand, vaccination with control Ad vaccines (3x10^8 ifu) did not have significant adverse effects on the chicken embryos, and the hatchability (>80%) in this group is acceptable (Fig. 19). This result suggests that the dose of Ad-(CD6-DAG1-Cpα$_{CD}$-FLAG) for *in ovo* vaccination needs to be optimized and lower doses (such as 3x10^6 ifu) may be more practical.

Compared to traditional field vaccine administration, *in ovo* vaccination helps close the window of susceptibility between early exposure to pathogens and post-hatch vaccination because both innate and adaptive immunity against specific pathogens were already stimulated in *in ovo* vaccinated embryos (Negash et al., 2004). Besides maternal antibody, *in ovo* vaccinated chicks have already developed an appreciable degree of protection by the time of hatch (Mesonero et al., 2011).

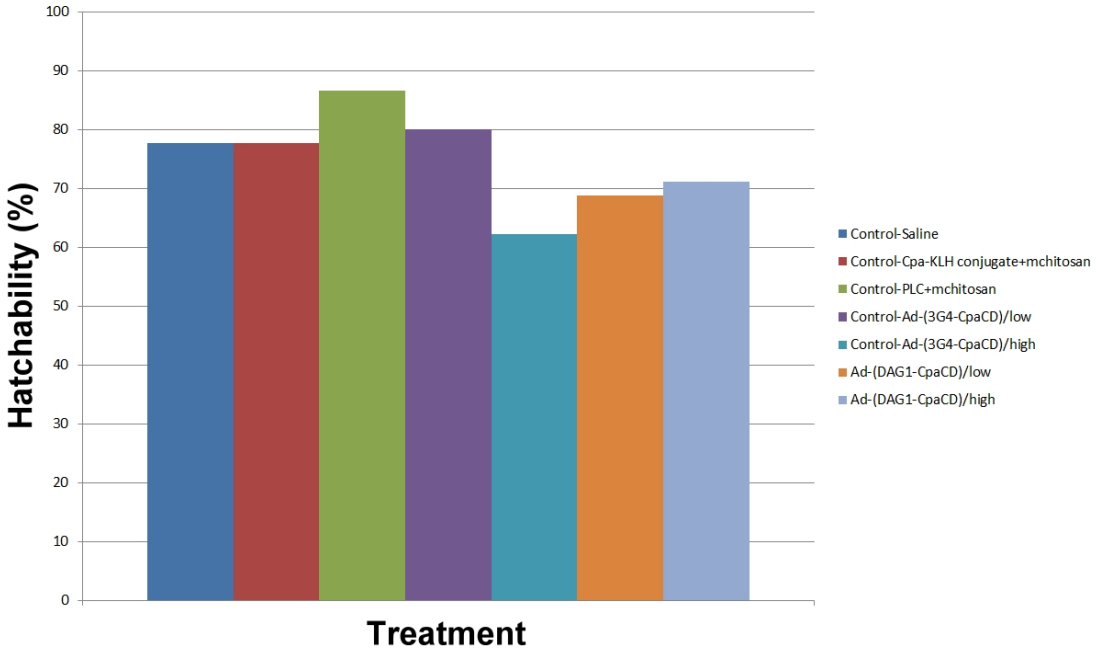

Fig. 19. Embryonated eggs vaccinated with Ad-(CD6-DAG1-Cpα$_{CD}$-FLAG) *in ovo* had hatchability identical to those from control groups.

We expected to observe robust mucosal immunity against Cpα and an improved IgA response in the Ad-(CD6-DAG1-Cpα$_{CD}$-FLAG) vaccinates as compared to controls. Besides Cpα peptide-KLH conjugate control group, chickens from other groups did not produce significant Cpα-specific IgA / IgG response (Fig. 20 and Fig. 21). Only chickens immunized *in ovo* with Cpα peptide-KLH conjugate mixed with modified chitosan produced a significant Cpα-specific IgG response ($P < 0.01$) 4 days post *C. perfringens* challenge (Fig. 21). The toxicity of recombinant adenovirus expressing the truncated Cpα$_{CD}$ when administered *in ovo* is not expected because toxicity of the alpha toxin is lost upon truncation (Williamson and Titball, 1993).

Tregaskes and colleagues (Tregaskes et al., 2005) reported that overdose administration of chCD154 on chicken APCs causes APCs to undergo apoptosis instead of differentiation or development *in vivo*. In the mouse model, overdose administration of

agonistic anti-mouse CD40 can also cause apoptosis on splenic B-cells both *in vivo* and *in vitro* (Qi et al., 2004; Gao et al., 2012). Since hatchability data suggest that Ad-(CD6-DAG1-Cpα$_{CD}$-FLAG) may have been over-administered to E18 eggs, the population of B-cells with BCRs, which recognized Cpα$_{CD}$, in GALT, especially Peyer's patch, and primary lymphoid organs may undergo apoptosis through the CD40 pathway, and this scenario can also be used to explain why no Cpα-specific antibody was found in chickens immunized with Ad-(CD6-DAG1-Cpα$_{CD}$-FLAG) post hatch. However, apoptosis in chicken APCs caused by super-optimal doses of DAG1 needs to be further elucidated.

Protection of chickens against *C. perfringens* challenge in Ad-(CD6-DAG1-Cpα$_{CD}$-FLAG) immunized chickens was assessed by body weight gains (Fig. 22), gross pathology at necropsy (Fig. 23), and mortality rate (Fig. 24) in the experiment. No significant increase in body weight gain was observed in chickens among all vaccine groups. Data of lesion scores and mortality also do not show any statistical difference among any of the vaccine groups. These results suggest that chickens immunized with Ad-(CD6-DAG1-Cpα$_{CD}$-FLAG) *in ovo* were not protected from *C. perfringens* challenge. Since no Cpα-specific antibody response, especially mucosal IgA, was detected in any bird post-vaccination besides Cpα peptide-KLH control group; protective effects against *C. perfringens* challenge were not expected at this point.

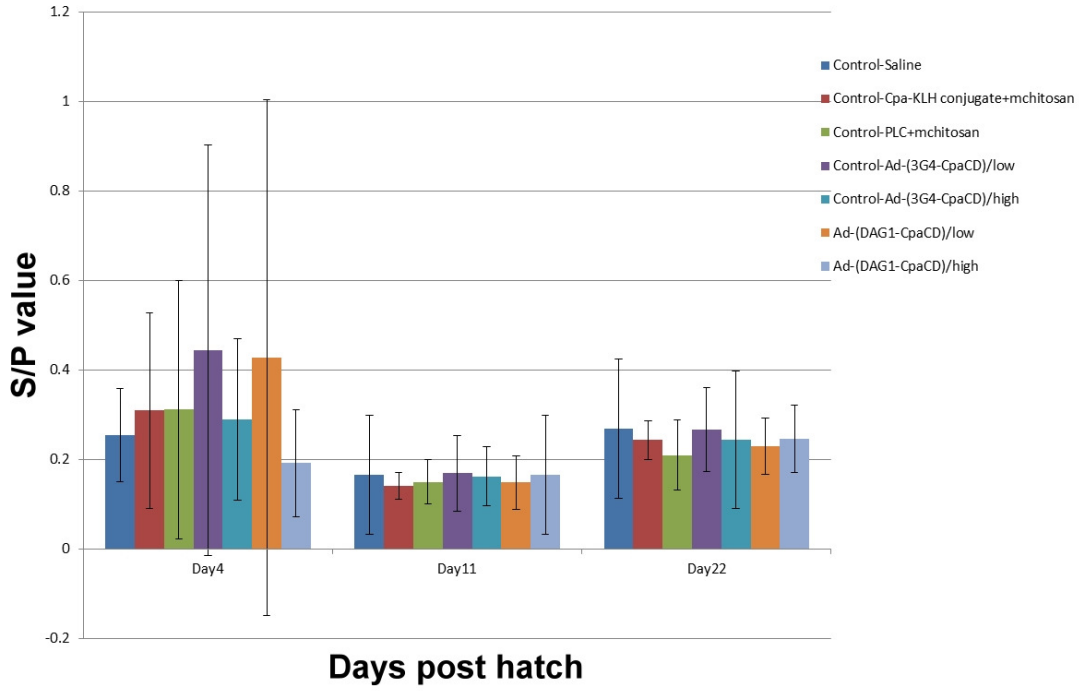

Fig. 20. Alpha-toxin specific mucosal IgA ELISA S/P values from Cobb 500 chickens immunized with different vaccines *in ovo* followed with challenge by *C. perfringens* Cp641 and Texas A&M strains. Trachea were collected at three time points: day 4, for determination of Cp*α*-specific IgA response after *in ovo* vaccination; day 11, mid-experiment for determination of pre-challenge Cp*α*-specific IgA response; and day 22, for determination of post-challenge Cp*α*-specific IgA response from chickens that survived the *C. perfringens* challenge. In each case, error bars represent standard deviations from the mean and the asterisks represent statistical significance (n=10; **P*<0.05; ***P*<0.01; ****P*<0.001) compared with Ad-(CD6-3G4$_d$-Cpα_{CD}-FLAG) control as determined by Student's t-test. Embryonated eggs vaccinated *in ovo* with control 1: Saline; control 2: Cp*α* peptide-KLH conjugate mixed with modified chitosan; control 3: Phospholipase C from *C. perfringens* mixed with modified chitosan; control 4: 3x10^8 ifu Ad-(CD6-3G4$_d$-Cpα_{CD}-FLAG)(low); control 5: 3x10^9 ifu Ad-(CD6-3G4$_d$-Cpα_{CD}-FLAG)(high); treatment 1: 3x10^8 ifu Ad-(CD6-DAG1-Cpα_{CD}-FLAG)(low); treatment 2: 3x10^9 ifu Ad-(CD6-DAG1-Cpα_{CD}-FLAG)(high)

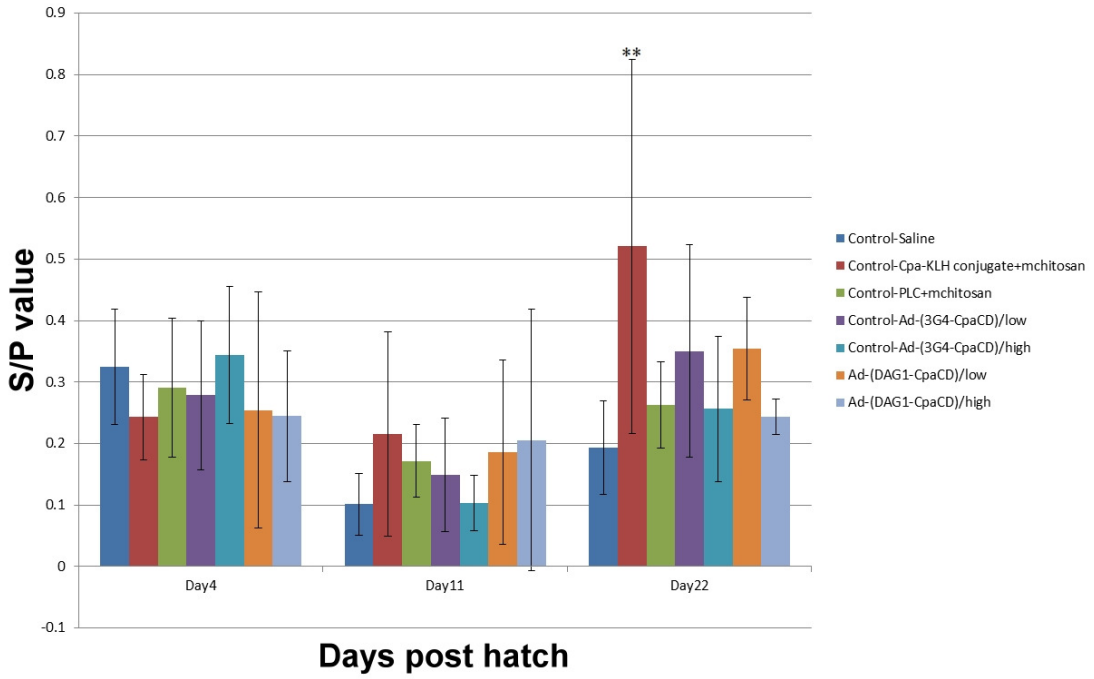

Fig. 21. Serum alpha-toxin specific IgG ELISA S/P values from all Cobb 500 chickens immunized with different vaccines *in ovo* followed with challenge by *C. perfringens* Cp641 and Texas A&M strains. Blood samples were collected at three time points: day 4, for determination of Cp*α*-specific IgG response after *in ovo* vaccination; day 11, mid-experiment for determination of pre-challenge Cp*α*-specific IgG response; and day 22, for determination of post-challenge Cp*α*-specific IgG response from chickens that survived the *C. perfringens* challenge. In each case, error bars represent standard deviations from the mean and the asterisks represent statistical significance (n=10; **P*<0.05; ***P*<0.01; ****P*<0.001) compared with Ad-(CD6-3G4$_d$-Cpα_{CD}-FLAG) control as determined by Student's t-test. Embryonated eggs vaccinated *in ovo* with control 1: Saline; control 2: Cp*α* peptide-KLH conjugate mixed with modified chitosan; control 3: Phospholipase C from *C. perfringens* mixed with modified chitosan; control 4: 3x10^8 ifu Ad-(CD6-3G4$_d$-Cpα_{CD}-FLAG)(low); control 5: 3x10^9 ifu Ad-(CD6-3G4$_d$-Cpα_{CD}-FLAG)(high); treatment 1: 3x10^8 ifu Ad-(CD6-DAG1-Cpα_{CD}-FLAG)(low); treatment 2: 3x10^9 ifu Ad-(CD6-DAG1-Cpα_{CD}-FLAG)(high)

For broiler producers, *in ovo* vaccination is an attractive approach for vaccination of great numbers of birds against devastating diseases, such as IBDV (Williams and Zedek, 2010). *In ovo* vaccination with recombinant adenovirus was demonstrated as an efficient method for controlling various infectious diseases, especially avian influenza (Breedlove et al., 2011). Another advantage of *in ovo* vaccination is fast (50,000 egg/hour) and uniform antigen delivery, and early protective immune responses can be elicited as early as the day of hatch. Compared with traditional field vaccination, using an *in ovo* vaccination strategy can greatly reduce labor cost and does not stress the birds.

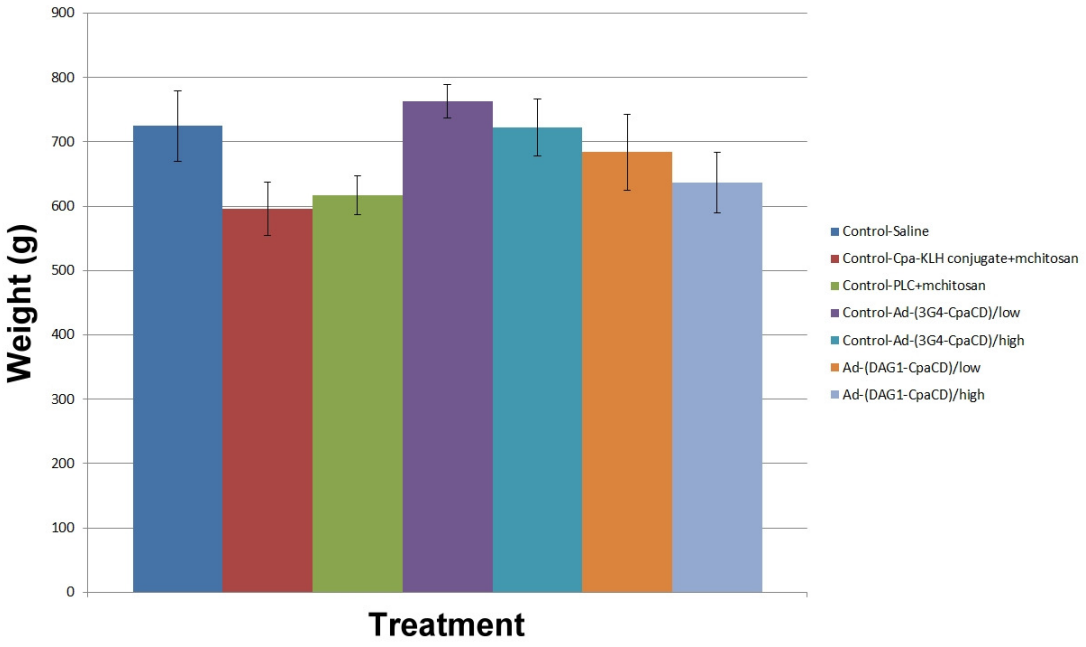

Fig. 22. Chickens immunized *in ovo* with Ad-(CD6-DAG1-Cpα$_{CD}$-FLAG) did not have statistically different body weight gain compared to control groups after *C. perfringens* challenge (*P* = 0.084).

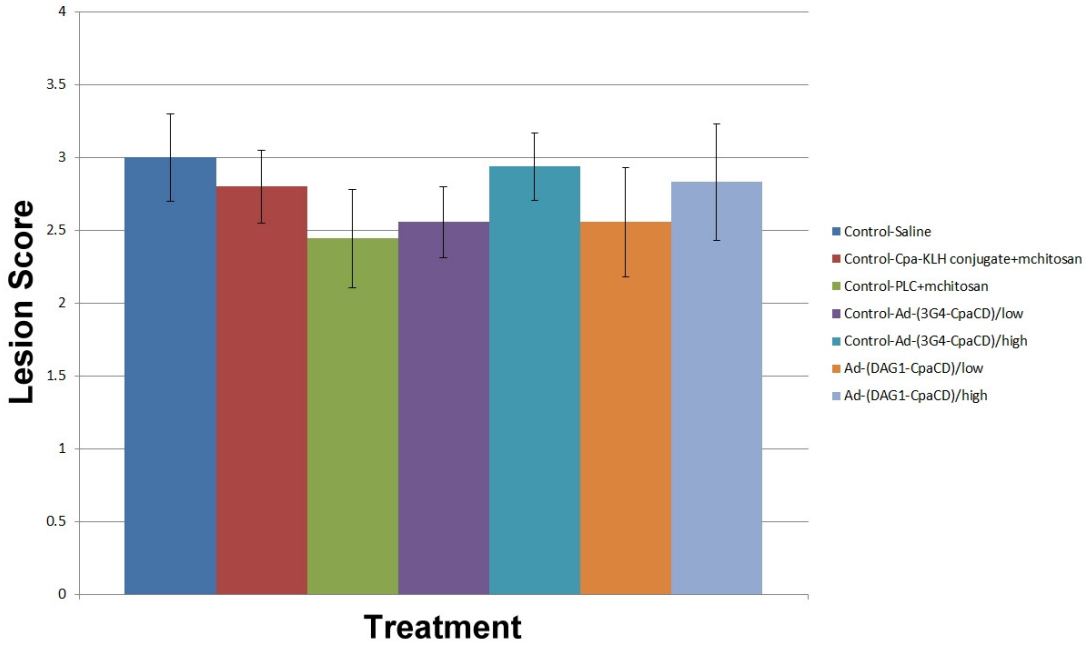

Fig. 23. Groups of chickens immunized *in ovo* with Ad-(CD6-DAG1-Cpα$_{CD}$-FLAG) did not have statistically significant lower lesion scores than control groups after *C. perfringens* challenge (*P* = 0.783).

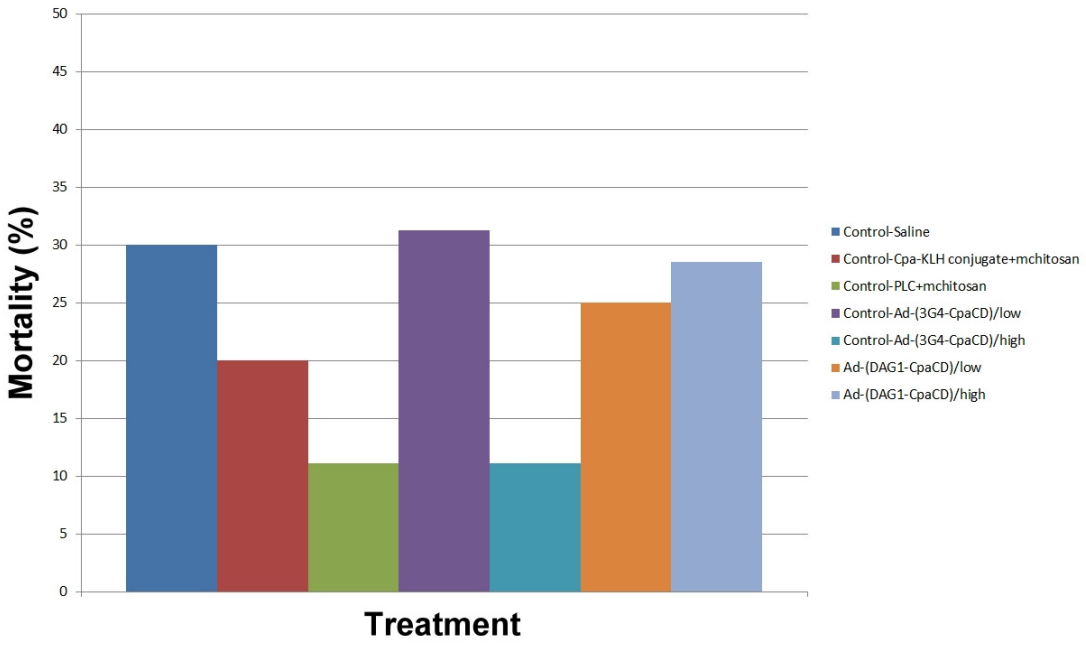

Fig. 24. Groups of chickens immunized *in ovo* with Ad-(CD6-DAG1-Cpα$_{CD}$-FLAG) at either dose showed higher mortality after *C. perfringens* challenge than control groups.

These preliminary data show that chickens vaccinated *in ovo* with Ad-(CD6-DAG1-Cpα$_{CD}$-FLAG) at neither 3 x10^8 nor 3 x 10^9 ifu do not produce enhanced IgA or IgG responses and were susceptible to NE by *C. perfringens* challenge. Nevertheless, it is still tempting to speculate that robust and quick protective responses against α-toxin might be obtained under the same settings while administering lower adenovirus quantities *in ovo* for more controlled and efficient antigen delivery to the APCs and enhanced stimulation of Cpα-specific B-cells. Another aspect in which this *in ovo* strategy can potentially be improved is to co-deliver another immunopotentiator, like chicken B-cell activating factor (chBAFF), by adenovirus. Ad5-chBAFF has potential as an immunopotentiator and IgA switch factor to also be used in combination with other *in ovo* or neonatal vaccines (Chen et al., 2009). Chicken BAFF (chBAFF) is a highly conserved cytokine that promotes the survival of splenic chicken B-cells *in vitro* (Schneider et al., 2004). In addition, recombinant chBAFF

induces the selective expansion of B-cells in the spleen and cecal tonsils when administered to young chickens (Schneider et al., 2004). The binding of recombinant human BAFF to chicken bursal B-cells also indicates conserved receptor ligand binding (Koskela et al., 2004). Finally, treatment of 3-day old chickens with chBAFF resulted in significantly increased antigen–specific mucosal IgA response compared to control birds (Kothlow et al., 2010), which strongly suggests that chBAFF functions as an isotype switching factor that promotes the production of mucosal sIgA, as is the case in mammals. Ad-chBAFF along with Ad-(CD6-DAG1-Cpα_{CD}-FLAG) should be used for *in ovo* vaccination to enhance Cpα-specific IgA responses at the mucosal surface and to thereby improve protection against *C. perfringens* challenge and thus NE.

CHAPTER VI

SUMMARY AND CONCLUSION

Summary

Necrotic enteritis (NE) is an economically devastating enteric disease of chickens primarily caused by infection with *C. perfringens* type A. Vaccination is currently the most promising control strategy for controlling NE. Therefore, there is strong interest in immunological adjuvants with low reactogenicity but high potency to enhance immune responses. Arguably one of the most successful strategies to attain this end consists of attaching the antigen to an antibody against a co-stimulatory cell surface receptor expressed by antigen-presenting cells (APC), such as CD40. Agonistic anti-CD40 antibodies not only target antigen delivery and activate B-cells, but also induce antibody class-switching on B-cells both *in vitro* and *in vivo*. The antibody class-switching is crucial because IgA is readily transported across the intestinal mucosa and is endowed with effector properties that are critical for the local humoral immune response. The central hypothesis here claims that *in ovo* vaccination with an adenovirus-vectored C-terminal domain of Cpα fused to an agonistic anti-chicken CD40 diabody vaccine capable of targeting antigen to APCs, which will improve mucosal IgA response and protection in chickens against NE. This hypothesis was tested through completion of the following specific experiments.

In the first experiment, an agonistic monoclonal anti-chicken CD40 antibody 2C5 was produced and characterized. Extracellular domain of chicken CD40 was cloned from chicken splenocytes and then expressed in FreeStyle™ 293 cells for monoclonal antibody production. The specific binding of antibodies secreted by anti-chCD40 hybridomas to chCD40 were

screened by immunocytochemistry, flow cytometry, and immunoprecipitation on chicken APCs. Positive clones were further selected for their agonistic activities on chicken APCs. Agonistic activities of anti-chCD40 mAbs were assessed by enhancement on cellular proliferation of serum starved DT40 B-cells and production of NO by HD11 macrophages. Among all positive clones, 2C5 is the most promising and suggested to mimic partial biological functions of chicken CD154, and thus has great potential as an immunological adjuvant.

In the second experiment, the potential of 2C5 as potentiator of a subunit vaccine consisting of a non-immunogenic peptide in five-week old chicks was assessed in an *in vivo* experiment. This 2C5 based vaccine complex was prepared by complexing biotinylated 2C5, streptavidin, and biotinylated peptide at a stoichiometric ratio of 2:1:2. Chicks (7 chicks / group) were immunized subcutaneously with 2C5-peptide complex at three dose levels (10µg, 30µg, and 90µg) in the nape of the neck. A single subcutaneous immunization with any dose of this 2C5 based vaccine complex was able to generate significant peptide-specific IgG responses ($P < 0.001$) as soon as 4 days through 2 weeks post immunization. This result demonstrated that CD40-targeted antigen delivery is an effective strategy to enhance the adaptive immune response to subunit vaccines in chickens in terms of speed, duration and isotype class switching.

A recombinant adenovirus expressing an agonistic anti-chCD40 single chain diabody (designed as DAG1) fused to C-terminal domain of *C. perfringens* α-toxin chimeric protein was tested for enhancement of mucosal IgA secretion and protection of chickens upon challenge while it was administrated by *in ovo*. No statistical significant difference was found on IgA / IgG response from any of vaccine groups during the experiment. After *C.*

perfringens challenge, lesion scores, body weight gain, and motality results conclude that no protection effect was found in chickens *in ovo* immunized with this recombinant adenovirus vaccine. Overdose administration of vaccine may be the reason why CD40 targeting hypothesis was rejected because overdose administration of agonistic anti-chCD40 diabody may cause chicken APCs, especially Cpα-specific B-cells, undergo apoptosis. The dose of recombinant adenovirus expressing DAG1-Cpα_{CD} for *in ovo* vaccination of E18 eggs needs to be optimized, and lower dose may be used for more accurate, controlled, and efficient stimulation on Cpα-specific B-cells and antigen delivery.

Conclusion

Specific binding of anti-chCD40 mAb to chCD40 was confirmed *in vitro* by immunocytochemistry and immunoprecipitation, and CD40 expression profile on chicken APCs was further confirmed by flow cytometry, indicating that 2C5 can be used for detailed screening of the expression level and distribution of chCD40 on various chicken cells and tissues. The identification of this potent activator of both chicken macrophages and B-cells will be useful in the further study of CD40L/CD40 interactions in the chicken, which is a valuable asset with regard to its potential use in vaccine development. Since accelerated, enhanced, isotyped-switched IgG response against a non-immunogenic peptide was only found in chickens immunized with 2C5-peptide complex, we conclude that this enhanced immunogenicity is due to the engagement of 2C5 with CD40 on APCs *in vivo*, by which directly activates peptide specific B-cells. This effect may be related to a better employ of this peptide by enhancing the engagement of 2C5 on the complex for B-cell activation simultaneously with CD40 on the surface of peptide-activated B-cells to induce the peptide-

specific IgG response. The anti-CD40 mAb-based adjuvant action is extremely potent and may avoid the inflammatory side effects induced by most classical adjuvants.

Chicken mucosal tissues are rich in APCs, we also expect that Ad-(CD6-DAG1-Cpα_{CD}-FLAG) could induce efficient systemic and mucosal immune responses through the *in ovo* immunization route. However, neither statistical significant IgA / IgG response nor protection against *C. perfringens* challenge was found in chickens *in ovo* vaccinated with Ad-(CD6-DAG1-Cpα_{CD}-FLAG). Our preliminary data suggests that over-dose administration of recombinant adenovirus may be the reason why no significant Cpα-specific IgA / IgG response was found in chickens *in ovo* vaccinated with Ad-(CD6-DAG1-CPα_{CD}-FLAG). The variety of contributions of CD40 targeting to chicken APCs through *in ovo* administration attest to the great versatility of the CD40-CD154 pathway in contributing to both normal immune responses and protection against T-cell independent / dependent antigens, as well as the dangers powerful CD40-mediated activating signals pose for the development and progression of chronic inflammatory diseases and apoptosis on chicken APCs.

The poultry industry is in urgent need of one-shot vaccines because it helps to save budget on fee from labor, time, and other overheads in traditional vaccination programs. Additionally, time for the production of protective antibodies in chickens can be shortening for several weeks by one-shot vaccine because of the enhanced adjuvant effects. This study has facilitated the design of approaches to apply CD40-targeting strategy while it attempts to improve the effects of subunit vaccines against a variety of chicken diseases. However, using optimized dose of Ad-(CD6-DAG1-Cpα_{CD}-FLAG) for *in ovo* vaccination is a tempting alternative control strategy for NE, which is efficacious, safe, and affordable.

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